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Biosynthesis and Characterization of Silver Nanoparticles Using Papaya leaves and Estimation of its Antibacterial Activity

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Abstract: ***In the present study, we aim in the biological synthesis of silver nanoparticles (AgNPs) using aqueous leaf extract of Carica papya. Bio reduction of Ag^+ to Ag^0 was observed when aqueous extract augmented with silver nitrate ($AgNO_3$) and kept at different reaction conditions (temperature, extract concentration). Reducing ability of the leaf extract of Carica papaya in the formation of silver nanoparticles (AgNPs) from silver nitrate was tested by determining the total phenolic content in papaya leaf, which act as reducing and capping agent. The formation of silver nanoparticles was primarily analysed by surface Plasmon resonance as determined by UV-VIS spectra ranging from 420-680 nm. Silver nanoparticles of size ranging 13-17 nm of spherical shape and smooth surface were characterized using transmission electron microscopy (TEM), which is the confirmatory analysis. Particles compositions were studied using FTIR spectroscopy. These nanoparticles were found to possess potential antibacterial activity against Escherichia coli with considerable zone of inhibitions by Oxford agar well diffusion method and turbidimetric measurements. This environmental friendly method provides a simple, easy, fast and cost effective method for nanoparticles synthesis and can be used in several areas of medicines including drug targeted delivery and gene therapy.***

Keywords: Escherichia coli, turbidimetric, surface Plasmon resonance, transmission electron microscopy

1. Introduction

Carica papaya belongs to the family of Caricaceae, and several species of Caricaceae have been used as remedy against a variety of diseases originally derived from the Southern part of Mexico. Carica papaya is a perennial plant, and it is presently distributed over the whole tropical area. In particular, Carica papaya fruit circulates widely, and it is accepted as food or as a quasi-drug. Many scientific investigations have been conducted to evaluate the biological activities of various parts of Carica papaya. The leaves of papaya have been shown to contain many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic, glucosides and glucosinolates.

Nanobiotechnology is playing an important role in bringing advances in scientific world. This technology has broad application in the fields including electronics, biomedical sciences, pharmaceuticals, cosmetics preparation, water filtration and catalytic systems[1]. Advances have been made in measuring sub cellular level and understanding the cell as highly organized, self-repairing, self-replicating and information rich molecular machines at nanoscale. Nanoparticles are used as the fundamental building blocks of nanotechnology. Nanoparticles (NPs) of noble metals like silver and gold also have potential application in various fields including biomedicine, where they can be used for drug and gene delivery systems and treatment of some cancers.

The synthetic methodologies of nanoparticles synthesis involve complex physical [2] and chemical processes [3,4] that use high temperature, high pressure, large amounts of energy and many toxic substances producing pollution to the environment. Major parameters for the synthesis of nanoparticles are the selection of solvent, reducing agent, nontoxic substances for the synthesis. Biological synthesis of nanoparticles proved to be cost effective means over chemical means as it does not involve physical barriers with regard to reducing agents and eliminates the toxic effects of chemicals used for the synthesis. At present, a number of living organisms are already known to synthesize nanoparticles such as cyanobacteria, bacteria, fungi [5], actinomycetes and various plant materials such as Cinnamomum camphora, Medicago sativa, Tamarindus indica, Parthenium hysterophorus, Sesuvivm potulacastrum [6] and gold nanoparticles also synthesized by biomolecules like Honey. Leaf extracts of Neem, Hibiscus, Cinnamon, Tamarind, Coriander and many plant and seeds such as Gram and maize have been used for development of nanoparticles. So, the living plants are considered as eco-friendly nanofactories.

The potential in vivo use of nanoparticles as antibacterial agents depends on their cytotoxicity and genotoxicity to eukaryotic cells [7]. Nanoparticles are widely used in bio applications, but the rapid progress and acceptance of nanobiotechnology cannot indicate the long-term impact on human health and the environment. The efficacy of silver nanoparticles depends on a particle's properties such as: size, shape, exposure time, the types of compounds, and target - and these properties have a significant impact on their biomedical efficacy. The biomedical efficacy of silver nanoparticles also depends on the sensitivity of pathogens; it results from the natural and purchased properties of cells (from structure and stoichiology). The pathogens of people, animals and plants are all sensitive to silver nano forms.

Research into the medical applications of silver nanoparticles has been extremely active. More and more innovative applications are being proposed and evaluated. One of these medical fields is the decrease of hospital-acquired infections during medical intervention by using

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bone and cardiovascular implants and catheters impregnated with silver nanoparticles. Silver nanoparticles satisfy the requirements by having an ideal antibacterial coating which displays: prolonged activity, a high level of bactericidal and bacteriostatic efficacy against a wide spectrum of microorganisms [8], and biocompatibility. Using silver nanoparticles as the coating of a catheter and eliminating hospital-acquired infections have great importance in the inhibition of biofilm formation and eradication.

It has been known for a long time that silver compounds are very effective antibacterial agents against both aerobic and anaerobic bacteria [9]. The use of silver in nanoparticle form (as compared to its ionic form) seems to have reduced cellular toxicity and antibacterial efficacy. Indeed, in one of the journal's most cited articles, Kim et al demonstrated clearly that the superior antibacterial properties of AgNPs are due to the formation of free radicals from the surface of silver. The antibacterial spectrum even extended to antibiotic resistant organisms.

Furthermore, the addition of antibiotics to AgNPs has been shown to have synergistic effects against micro-organisms. Apart from being an excellent anti-bacterial agent, AgNPs appears to have anti-inflammatory properties as well. Nadworny et al explored the effect of AgNPs using a porcine model of contact dermatitis. Here, it was confirmed that AgNPs had direct anti-inflammatory effects and improved the healing process significantly when compared with controls. Addition of AgNPs reduced the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-alpha and interferon-gamma, although the intracellular pathways involved still remains largely not elucidated.

2. Materials and Methods

2.1 Sample Collection:

The Carica papaya leaves were collected from a 1 year old papaya plant from Chemboor (Thiruvananthapuram). Nearly 6 to 10 mature leaves were taken. They were first washed with tap water and then by distilled water.

2.2 Preparation of Aqueous Extract:

200g of papaya leaves were ground using mortar and pestle to form clear paste (by adding distilled water). 25 ml crude leaf paste was diluted 5 times with distilled water. The filtrate thus obtained by aqueous extraction was then subjected to hot percolation and cold percolation treatment. 50ml of 1mM silver nitrate was taken as control for the experiment.

In hot percolation treatment, 50ml leaf paste diluted with distilled water was taken in 250ml conical flask and stirred at 40˚C for 2 hours. The resultant mixture was then filtered out using Whattman filter paper No.1 (pore size 25µm) and then the filtrate was kept in water bath at 60˚C till reduced volume of filtrate was obtained.

In cold percolation treatment, 50ml leaf paste diluted with distilled water was taken in 250ml conical flask and kept in shaking incubator for 24 hours at 30˚C. After incubation resultant mixture was filtered out by using Whattman Filter Paper No.1 (pore size 25µm).The filtrate so obtained from hot percolation and cold percolation treatment were used as raw extract for the synthesis of silver nanoparticles.

2.3 Biosynthesis of Silver Nanoparticles

In different 250ml conical flasks, 2.5 ml of extract with 50 ml of 1mM $AgNO_3$ were added and kept at different reaction temperatures: 4, 20, 37and 90˚C for 3 hours. It was carried out for both hot and cold percolation extract. Similarly we add 50ml of 1mM $AgNO_3$ and different extract volume (0.5ml, 2.5ml, 4.5 ml) in different conical flasks, and kept at 37˚C for 3 hours. It was also done for both cold percolation and hot percolation extract.

Figure 1: Samples for temperature variation at 4℃, 20℃, 37℃, 90℃

Figure 2: Samples for extract volume variation of 0.5, 2.5, 4.5 ml

2.4 Identification and Characterization of Biologically Synthesized Silver Nanoparticles

2.4.1: Color Change:

Change in colour of leaf extract from dark green to brownish red was observed. This colour change preliminary showed the presence of silver nanoparticles or reduction of Ag^+ of $AgNO_3$ to Ag^0.

2.4.2: UV-VIS Analysis:

UV-VIS analysis was a preliminary analysis for the presence of silver nanoparticles. The samples were scanned from 420-680 nm and absorbance was recorded UV-VIS spectrophotometer.

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2.4.3: Transmission Electron Microscopy (TEM) Analysis:

Analysis was carried out by TEM analysis. Aqueous sample with maximum absorbance was given to Sree Chithira Research Institute for TEM analysis.

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

2.4.4: Fourier Transform Infrared Spectroscopy (FTIR):

was carried out for the characterization of nanoparticles. Sample (aqueous) with maximum absorbance was given to National Institute for Interdisciplinary Science and Technology for FTIR analysis.

Fourier Transform Infrared Spectroscopy FTIR is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas. An FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time.

2.5: Estimation Of Total Phenolic Content

Reducing ability of the leaf extract of Carica papaya in the formation of silver nanoparticles (AgNPs) from silver nitrate was tested by determining the total phenolic content in papaya leaf, which act as reducing and capping agent. Phenolics are secondary plant metabolites that have been shown to contain high levels of antioxidant activities. Phenolic compounds act as free radicals scavengers due to their hydroxyl groups which contribute directly to the antioxidative action or reducing action.

Procedure

200ml of aqueous samples of silver nanoparticles and crude leaf extract were centrifuged at 3000 rpm for 30 min. Silver nanoparticles and leaf extract precipitated at the bottom of the centrifuge tube, they were collected in a petriplate and dried in the oven at 50˚C.1g of dried silver nanoparticle powder and leaf extract powder were obtained.

Standard: 1ml aliquots of 20,40,60,80,100,120 and 140µg/ml of aqueous gallic acid was made up to 5ml using distilled water.

Test sample: 10mg of papaya leaf extract and 10mg of silver nanoparticles was dissolved in water to get the appropriate concentration (1 mg/ml). 1.0 ml of each extract in a test tube was mixed with 5.0 ml of distilled water.

1.0 ml of Folin-Ciocalteau reagent was added and mixed thoroughly. 3 min later, 3.0 ml of saturated sodium carbonate solution was added and the mixture was allowed to stand for 90 min in the dark.The absorbance of the colour developed was read at 725 nm using UV–Vis spectrophotometer.

The concentration of total phenolic content in the extracts was determined as µg ofgallic acid equivalent (GAE) by standard calibration curve. Three replicates were performed for each sample concentration to check the reproducibility of the experimental result and to get a more accurate result.

2.6: Evaluation of Antibacterial Activity:

The antibacterial activities of the silver nanoparticles were determined by oxford agar well diffusion method and turbidimetric measurement.

2.6.1: Oxford Agar Well Diffusion Method:

Medium Preparation- Nutrient Agar

500ml of nutrient agar was prepared by taking 2.5g peptone, 1.5g beef extract in 250ml of distilled water and boil the mixture. 7.5g agar was separately boiled in distilled water and is added to the above constituents. The above mixture was made upto 500ml using distilled water and boil the mixture till the constituents were homogenized then it was sterilized using autoclave at 121˚C, 15 psi for 20 min.

Plate Preparation

15 ml of nutrient agar were poured into the petriplate. After the soldification of nutrient agar plates, it was inoculated with 0.5ml of E.coli culture. The plate was divided into 4 quadrants. 3 wells were made by a sterile cork borer of 10mm diameter in 3 quadrants and standard antibiotic streptomycin disc (10mg/disc) was placed on the agar surface of 4th quadrant. 100 microliters of silver nitrate, silver nanoparticles and crude papaya leaf extract were added.

2.6.2 Turbidimertic Measurement

Medium preparation-

MH broth 500ml of Mueller-Hinton (MH) broth was prepared by adding 1g Beef extract, 8.75g Casein and 0.75g Starch in 250ml distilled water and made up to 500ml and was sterilized by autoclave at 121˚C, 15 psi for 20min.

Preparation of samples:

200ml of aqueous samples of silver nanoparticles were centrifuged at 3000 rpm for 30 min. Silver nanoparticles were pelletized at the bottom of the centrifuge tube.

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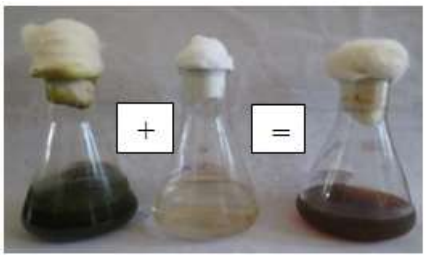
Pelletized samples were collected in a petriplate and dried in the oven. 80mg of dried silver nanoparticle powder were obtained.

5ml of MH broth was taken in 6 boiling tubes and added with different concentrations of silver nanoparticle powder (0, 2, 4, 6, 8 and 10mg) and 6th tube was taken as control. 0.5ml E.coli culture was inoculated in 6 boiling tubes. Each culture was then incubated in a shaking incubator at 37˚C, for 24 hours. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 610 nm.

3. Results

3.1 Color Change

The color change indicated that the addition of 50ml of 1mM silver nitrate ($AgNO_3$) to the crude Carica papaya leaf extract ,subjected to various reaction conditions, that is temperature and extract volume variations resulted in brown colored solutions; indicating the biosynthesis of silver nanoparticles.



Crude Leaf + Silver Nitrate = Nanoparticles

Figure 3: Synthesis of silver nanoparticles using papaya leaf extract and silver nitrate

3.2 UV-VIS Spectro - Photometric Analysis

The samples when treated with different reaction conditions, change in color of extracts suspension from dark green to brownish red were observed. This color change preliminary showed the presence of silver nanoparticles or reduction of Ag^+ to Ag^0. It is well known that silver nanoparticles exhibit yellowish brown color in water, which arises due to the excitation of Surface Plasmon Resonance in the metal nanoparticles. Metal nanoparticles such as silver have free electrons, which give rise to Surface Plasmon Resonance absorption band. After observing changes in color of the extracts, they were scanned from 420-680 nm in and maximum absorbance was observed at 440nm for samples obtained from cold percolation. Neither yellowish-brown color change in the reaction vessel nor a strong Plasmon Resonance peak was observed for the silver nitrate solution.

(i) Study of effect of temperature: It was interpreted that the sample containing 2.5 ml of leaf extract and 50ml of 1mM $AgNO_3$ incubated at 37^{0}C temperature obtained from cold percolation treatment has more number of silver nanoparticles as compared to hot percolation treatment .It was also observed that, with increase in temperature conditions from 4^{0}C to 37^{0}C, there was increase in number of silver particles were observed due to Surface Plasmon Resonance (SPR).

Figure 4: Samples after hot percolation treatment incubated for 3hours at 4^{0}C, 20^{0}C, 37^{0}C and 90^{0}C

The absorbance of the plant extracts subjected to hot and cold percolation treatments at temperature variations were recorded in table 1. On comparing the absorbance values of samples from hot percolation and cold percolation, there was an increase in the values with temperature upto 37˚C and maximum absorbance (3.079) was obtained from cold percolation treatment at 37˚C, at 440nm.

Table 1

Samples obtained from hot percolation treatment	Wavelength at which maximum absorbance obtained(nm)	Maximum absorbance (%)
AT4^{0}C	420	0.535
AT20^{0}C	420	0.253
AT37^{0}C	420	0.702
AT 90^{0}C	420	0.664
Samples obtained from cold percolation treatment	Wavelength at which maximum absorbance obtained(nm)	Maximum absorbance (%)
AT4^{0}C	425	1.184
AT20^{0}C	420	1.491
AT37^{0}C	440	3.079
AT 90^{0}C	475	3.020

(i) Study of effect of extract volume variation:

It was interpreted that raw extracts with extract volume variation 2.5ml prepared from cold percolation treatment produced more number of silver nanoparticles as those compared to hot percolation treatment .It was observed that the change in color and absorbance increase due to SPR.

Figure 5: Samples after hot percolation method incubated for 3hours at varying extract volume 0.5ml, 2.5 ml, 4.5ml

The absorbance of the plant extracts subjected to hot and cold percolation treatments at extract volume variations were recorded in table 3. On comparing the absorbance

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values of samples from hot percolation and cold percolation, there was an increase in the values with extract volume variation upto 2.5ml and maximum absorbance (3.079) was obtained from cold percolation treatment at 37˚C, at 440nm.

Table 2

Samples obtained from hot percolation treatment	Wavelength at which maximum absorbance obtained(nm)	Maximum absorbance (%)
0.5ml	**440**	**0.221**
2.5ml	**435**	**0.783**
4.5ml	**420**	**1.775**
Samples obtained from cold percolation treatment	**Wavelength at which maximum absorbance obtained(nm)**	**Maximum absorbance (%)**
0.5ml	420	0.397
2.5ml	440	3.097
4.5ml	450	3.000

The graph of OD vs. wavelength of the test sample that showed maximum absorbance at 37^0C(temperature variation) and 2.5ml(volume variation) obtained after cold percolation treatments were plotted:

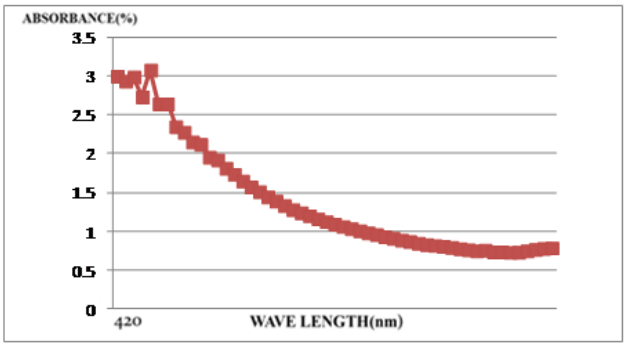

Figure 6: Absorption spectra of test sample at 37 ^{0} C (2.5ml)

3.3 Transmission Electron Microscopic Analysis

Sample from cold percolation with extract volume 2.5ml at 37℃ got maximum absorbance at 440nm.This sample is given for TEM analysis at Sree Chithira Research Institute.TEM analysis is confirmatory technique applied for identification of silver nanoparticles synthesized from plant extracts. Here TEM images showed that nanoparticles produced are mostly spherical in shape and their size varies 13nm. The Magnified images were observed by transmission electron microscope.

Figure 7: Magnified images of silver nanoparticles of size 13nm observed through Transmission Electron Microscopy (TEM).

3.4 Fourier Transform Infrared Spectroscopic Analysis

Sample from cold percolation with extract volume 2.5ml at 37˚C got maximum absorbance at 440nm.This sample is given for FTIR analysis at National Institute of Interdisciplinary Science and Technology. Maximum numbers of peaks were obtained in the range from 1238cm^{-1} to 1698cm^{-1}in the test sample (5) when compared to the control (4). This indicates the symmetric stretching of amino groups of amino acid residues present in the solution. The spectrum shows the bio-reduction of silver ions to silver nanoparticles is due to the reduction by the proteins present. They are the capping material in the reaction solution.

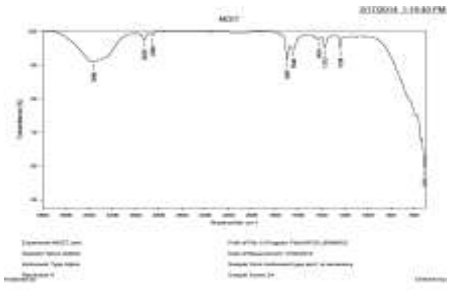

Figure 8: FTIR spectrum of samples containing biosynthesized silver nanoparticles.

3.5 Total Phenolic Content

The reducing capacity depends on the amount of water soluble phenolic compound present in the extract. During the reaction with silver nitrate, the phenolic compound donates electron to Ag^+ to produce Ag^0. After donation of structure of the same. The bioreduction of silver ions and the formation of AgNPs are closely related to the biomolecular component of the extract. An electron, the phenolic compounds changed into quinine which is stabilized by the resonance.

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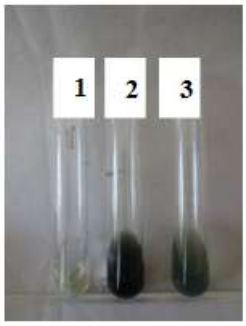

Figure 9: Estimation of total phenolic content

1. Control
2. Crude leaf extract
3. Silver nanoparticle

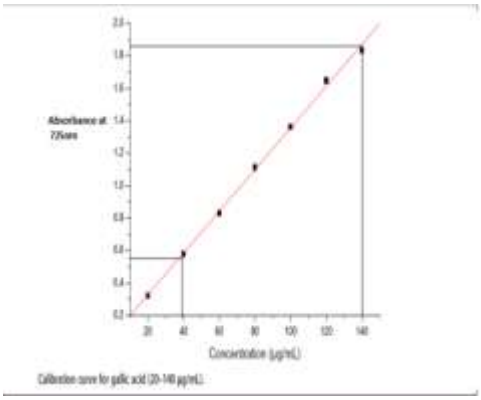

Figure 10: Calibration curve for gallic acid

Table 3

Sl no.	Sample name	Total phenolic content in the sample (µg/ml)
T_1	Papaya leaf extract	136
T_2	Silver nanoparticles	38

The absorbance showed that the total phenolic content in crude leaf extract was greater when compared to silver nanoparticles. It was interpreted that the formation of nanoparticle from silver nitrate is due to the reducing ability of phenolic content in papaya leaf extract.

3.6 Antibacterial Analysis

Silver nanoparticles so produced from papaya leaf extracts were assayed for their potential antimicrobial activity by the following methods .These nanoparticles showed antibacterial activity against Escherichia coli

3.6.1 Oxford Agar-Well Diffusion Method

Antibacterial assay was carried out and 10mm zone of inhibition was obtained for silver nanoparticles. Silver nanaoparticles exhibited higher antibacterial activity compared to that of crude leaf extract and silver nitrate, which was comparable with that of standard streptomycin antibiotic.

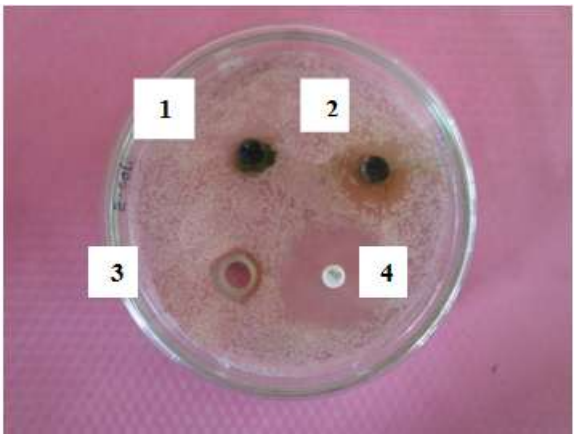

Figure11: Agar plated with

1. Crude leaf extract
2. Silver Nanoparticles
3. Silver nitrate 4. Streptomycin

3.6.2 Turbidimetric Analysis:

Turbidity testing determined the cloudiness of the Muller Hinton broth, measuring the loss of intensity of light beam through these solutions. The broth was inoculated with Escherichia coli and suspended with different concentrations of silver nanoparticle.

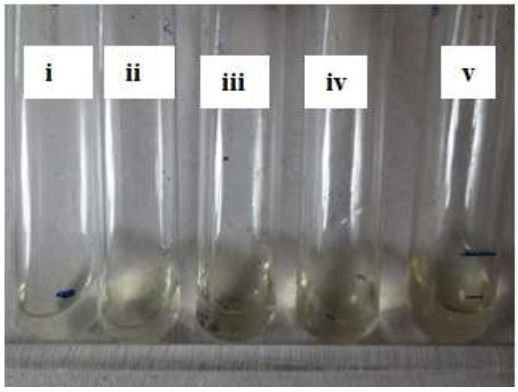

Figure11: The cloudiness that was observed after 24hours incubation in shaking incubator.

i. 2 mg silver nanoparticles/5ml MH broth
ii. 4 mg silver nanoparticles/5ml MH broth
iii. 6 mg silver nanoparticles/5ml MH broth
iv. 8 mg silver nanoparticles/5ml MH broth
v. 10mg silver nanoparticles/5ml MH broth

The absorbance of samples after 24 hours incubation in shaking incubator was measured at 610nm and was recorded in table 4:

Table 4

AgNP Powder (mg)	Absorbance (%)
0	0.924
2	0.854
4	0.823
6	0.774
8	0.701
10	0.621

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Minimum Absorbance was observed in sample with 10mg of silver nanoparticle/5ml of MH broth when inoculated with E.coli. This is due to minimum cloudiness or turbidity that indicates silver nanoparticles inhibit the growth of E.coli in MH broth. The curve for absorbance at 610nm vs silver nanoparticle concentrations was plotted and is given in figure 24.

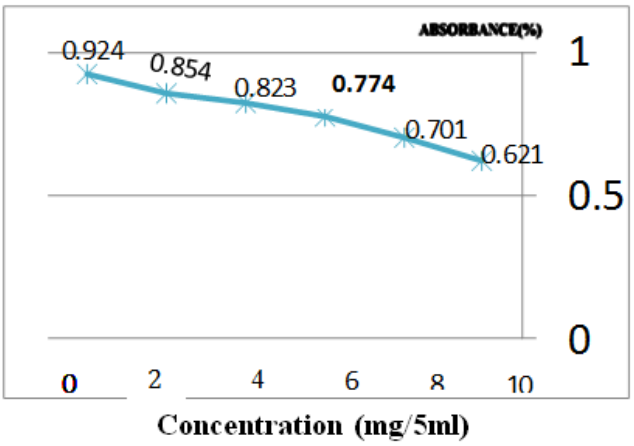

Figure12: Curve of turbidimetric analysis

From this, we conclude that silver nanoparticles have potential antibacterial activity.

4. Discussion

In this project, "Biosynthesis and characterization of silver nanoparticles using the leaf extract of Carica papaya and estimation of its antibacterial activity", the papaya leaf extract was prepared by hot and cold percolation treatment [10] and was analysed that cold percolation method was more effective than hot percolation method. The reducing property of aqueous leaf extract of Carica papaya was estimated by its total phenolic content. This bioactive component contributes to the reducing property, which plays a key role in the conversion of Ag^{+} of $AgNO_3$ to Ag^{0}[12].

Characterization of biosynthesized silver nanoparticles was carried out by preliminary and confirmatory analysis. The preliminary analysis was done by analyzing the color change and UV-VIS spectra, scanned from 420- 680nm. The maximum absorbance value was obtained from the sample at 37^{0} C, 2.5ml subjected to cold percolation treatment. The confirmatory analysis were carried out by Transmission Electron Microscopy (TEM)and FTIR [11]. TEM analysis determined that the silver nanoparticles are spherical in shape, having smooth surface and size ranging from 13-17 nm. FTIR results interpreted the composition of silver nanoparticles.

The main application of biosynthesized silver nanoparticles is their potential antibacterial activity. The Oxford Agar Well Diffusion method [11] and the turbidimetric method[13] showed that the biosynthesized silver nanoparticles showed higher potential against antibacterial activity when compared with the crude leaf extract of Carica papaya.

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