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Eco Freinfly Synthesis And Characterization Of Silver Nanoparticles Using Aquous Leaf Extract Of *Alphonsea Sclerocarpa* And Apply It As Antimicrobial

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Abstract:

To develop a reliable, eco-friendly and easy process for the synthesis of silver nanoparticles using leave extracts of medicinal plant "*Alphonsea sclerocarpa*". There has been an exponentially increasing interest in biological synthesis of AgNPs. In this study, AgNPs were synthesized by an eco-friendly and convenient method using *Alphonsea sclerocarpa* leaf extract at ambient temperature. *Alphonsea sclerocarpa* leaf extract has been used as a reducing agent for the synthesis of silver nitrate into silver nanoparticles. Green synthesized silver nanoparticles are confirmed by color change which was monitored quantitatively by UV-Vis spectroscopy at 440 nm (Fig.4). Further characterization with SEM, TEM and AFM analysis shows the spherical, polydisperse AgNPs of particle size ranging from 5 to 50 nm with an average size of 28.5 nm (Fig.7,8,9). FTIR showed the structure, the respective bands of the synthesized nanoparticles, and the stretch of bonds. The cytotoxicity analysis of the green synthesized silver nanoparticles

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was observed that showed highest antimicrobial activity against gram positive bacteria *Staphylococcus aureus* (MTCC-6908) (Fig.11) showed average zone of inhibition of (26 mm), the Gram negative bacteria *Pseudomonas citronellolis* (MTCC-1191) (Fig.12) showed average zone of inhibition of (18.3 mm) and the Fungi *Candida albicans* (MTCC-4748) (Fig.13) showed average zone of inhibition of (22.2 mm) (Fig.10). However, further investigations were needed to identify the scaling-up usage of this extract on metallic nanoparticle synthesis and its applications as antimicrobial.

INTRODUCTION:

Nanotechnology is a vital field of cutting edge research managing synthesis, procedure and manipulation of molecule's, sub molecule's and atoms structure extending from roughly 1-100 nm in size [1]. Inside this size range every one of the properties (chemical, physical and biological) [2-6] changes in primary ways for both individual molecules/atoms and their relating bulk. Novel purposes of nanoparticles and nanomaterials are becoming quickly on different fronts because of their totally new or improved properties based on scale, their distribution and morphology [7,8].

Nanobiotechnology is a branch of science that dealing between nanoscience and biotechnology involving the application of biological systems for the production, manipulation and design of new functional nano-scale materials [9]. It combines biological methods with physical and chemical procedures to generate nano-scale particles with unique functions. The synthesis of nanoparticles using green technology is advantageous over chemical agents owing to their environmental anxieties. There is significant to produce inorganic nanoparticles as they supply greater material properties with effective resourcefulness [10].

Nanobiotechnology is presently one of the most dynamic disciplines of research in contemporary material science whereby plants and different plant products are finding an imperative use in the synthesis of nanoparticles (NPs) [5,6].

Nanomedicine is the medical application of nanotechnology [11]. Nanomedicine ranges from the medical applications of nanomaterials and biological devices, to nanoelectronic biosensors, and even possible future applications of molecular nanotechnology such as biological machines [11].

Nanotechnology is swiftly gaining renovation in a large number of fields such as health care, cosmetics, biomedical, food and feed, drug-gene delivery, environment, health, mechanics, optics, chemical industries, electronics, space industries, energy science, catalysis, light emitters, single electron transistors, nonlinear optical devices, photo-electrochemical applications and many more to count on [12].

MATERIALS AND METHODS:

Alphonsea sclerocarpa plant:

Scientific classification:

Kingdom: Plantae

(Unranked): Angiosperms

(Unranked): Magnoliids

Order: Magnoliales

Family: Annonaceae

Genus: Alphonsea

Species: ***A. Sclerocarpa*** [13]

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Fig. 1 . *Alphonsea sclerocarpa* plant.

General description:

Alphonsea sclerocarpa is small trees in size 10-15 m, braches rugose, glabrous; branchlets drooping. Leaves simple, alternate, distichous, estipulate; petiole 6-8 mm long, slender, glabrous; lamina 3.5-10 x 1.5-4 cm, oblong, oblong-lanceolate, elliptic ovate; base cuneate or attenuate; apex obtuse, entire, coriaceous, glabrous; lateral nerves 5-10 pairs, slender, pinnate, prominent; intercostae reticulate as showen in (Fig. 1) [14-18].

Culture Media:

Growth Media NO. 3 (Nutrient Agar Medium):

To prepare Growth Media NO. 3 (Nutrient Agar Medium) by dissolving 1.0 g of Beet extract and 2.0 g of Yeast extract and 5.0 g of Peptone and 5.0 g sodium chloride NaCL powder and 15.0 g

of Agar in 1.0 L of distilled water and mix it properly till homogeneity of the liquid and finally sterilizes it in Autoclave for 15 min in 15 lb and 121 C^o [19].

Growth Media NO. 5 (YRPO Medium):

To prepare Growth Media NO. 5 (YRPO Medium) by dissolving 3.0 g of Yeast extract powder and 10.0g of Peptone and 20.0g Dextrose and 15.0 g of Agar in 1.0 L of distilled water and mix it properly till homogeneity of the liquid and finally sterilizes it in Autoclave for 15 min in 15 lb and 121 C[20].

Growth Media NO. 11 (MRS Medium):

To prepare Growth Media NO. 11 (MRS Medium) by dissolving 10.0g of Beet extract powder and 5.0 g of Yeast extract and 10.0g of Peptone and 20.0 g of Glucose and 2.0g of Na2HPO4 and 5.0g of Sodium Acetate and 2.0g of Triammonium citrate and 0.2g MgSO4.7H2O and 0.2g of MgSO4.4H2O and 15.0 g Agar and 1.0 ml of Tween 80 liquid in 1.0 L of distilled water and Adjust the PH at 6.2-6.6 and mix it properly till homogeneity of the liquid and finally sterilizes it in Autoclave for 15 min in 15 lb and 121 Co [21].

Growth Media NO. 65 (Malt Extract Agar Medium):

To prepare Growth Media NO. 65 (Malt Extract Agar Medium) by dissolving 3.0g of Malt extract powder and 3.0 g of Yeast extract and 5.0g of Peptone and 10.0 g of Glucose and 20.0 g Agar in 1.0 L of distilled water and Adjust the

PH at 6.2 and mix it properly till homogeneity of the liquid and finally sterilizes it in Autoclave for 15 min in 15 lb and 121 C^o [22].

Microorganisms Collection:

Provided us with bacterial and fungal cultures from Microbial Type Culture Collection Institute of Microbial technology Sector 39- A, Chandigarh-160036, by Mineral oil Preservation method at room temperature. Mineral oil Preservation method by prepare tubes of heart infusion agar with a short slant. For fastidious organisms, add fresh native or heated blood. Sterilize mineral oil (liquid petrolatum) in hot air (170 C^o for 1 hour). Grow a pure culture on the agar slant. When good growth is seen, add sterile mineral oil to about 1 cm above the tip of the slant. Subculture when needed by scraping growth from under the oil. Store at room temperature. Transfer after 6-12 months.

As mentioned bellow:

3.3.1 *Bacillus subtilis* - (MTCC No. 10407)

3.3.2 *Staphylococcus aureus* - (MTCC No. 6908)

3.3.3 *Pseudomonas citronellolis* - (MTCC No. 1191)

3.3.4 *Klebsiella pneumoniae* - (MTCC No. 9024)

3.3.5 *Lactobacillus acidophilus* - (MTCC No. 10307)

3.3.6 *Enterobacter aerogenes* - (MTCC No. 111)

3.3.7 *Candida albicans* - (MTCC No. 4748)

3.3.8 *Aspergillus niger* - (MTCC No. 9687)

Preparation of leaf Extracts:

Prepare Aqueous Extracts (Water):

The fresh and tender leaves of *Alphonsea sclerocarpa* were collected from the South India. These were thoroughly washed with tap water and kept under shade at room temperature for about (20) days till they dried completely. The dried leaves were mechanically grinded and sieved to get fine powder. The (5 g) of powdered leaves was mixed with (100) ml of distilled water, the mixture were boiled in the stirrer heat for 10 min in order to dismantle tearing the wall of plant cells, and then nominate the mixture through suppress and bowl funnel using the nomination papers (Whitman No.1). And this is prepared crude aqueous extract and stored in a refrigerator at 4 °C [23].

Preparation 1/1000 M of Silver Nitrate ($NaNO_3$):

Prepared (1 Mm) of Silver Nitrate (NaNO3) by waited (0.16987 g) of Silver Nitrate (NaNO3) and dissolved it with 1 L distilled water in Broun bottle (because it is sensitive to the light) in room temperature.

Silver Nanoparticle (Ag NPs) Synthesis:

The aqueous solution of (1 mM) silver nitrate (AgNO3) was prepared to synthesize AgNPs. (190 mL) of aqueous solution of (1 mM) AgNO3 was slowly added to (10 mL) of *Alphonsea sclerocarpa* aqueous leaf extract while stirring, for reduction into Ag ions and kept at room temperature, the emulsion turned to dark brown after 30 min. This confirmed the synthesis of AgNPs in the mixture solution. It is well known that silver nanoparticles exhibit dark brown color in aqueous solution due to excitation of surface Plasmon vibrations in silver nanoparticles.

UV-vis spectra analysis Samples (1 mL) of the suspension were collected periodically to monitor the completion of bioreduction of Ag+ in aqueous solution, followed by dilution of the

samples with 2 ml of deionized water and subsequent scan in UV-visible (vis) spectra, between wave lengths of 200 to 700 nm in a spectrophotometer. UV-vis spectra were recorded at intervals of 1 hr, 24 hr and 72 hr.

Cultivation of Microorganisms:

Cultivation of Bacteria:

Preparation of subculture by broke the head of the tube that provided us and remove the cotton and mix the contains with I ml distilled water and taken amount of the sample by sterilized loop and spread it on pretty dishes which have sterilized nutrient media agar types (3,5,11 and 65).

Cultivation of Fungus:

Preparation of subculture by broke the head of the tube that provided us and remove the cotton and mix the contains with I ml distilled water and taken amount of the sample by sterilized loop and spread it on pretty dishes which have sterilized nutrient media agar type (3).

Sterilization of Aqueous Extract:

It was prepared water extract, Hexane Extracts, Chloroform Extracts and Methanol Extracts and Sterilized the mixture by pasteurization method (temperature (62 ^{o}C) for a period of (10 minutes) and it was getting on the benchmark center for alcoholic extracts.

Prepare Silver Nanoparticles Disks:

By using filter paper (Whitman No. 1) and make disks from it by using piercer papers and concentrations from the liquid of silver nanoparticles and submersing the disks inside each concentration for 1 hr and then the disks taken out to dry and to using it as

antimicrobial against the bacteria and fungi.

Determination of antimicrobial activity:

Has been relying on the way researcher Bauer et al (1966) to test the sensitivity of the silver nanoparticles as antimicrobial against both of the bacteria and fungi, and then inoculating the surface of nutrient broth (4-5) pure colonies of bacteria under study, and incubated germ in degree (37 ^{o}C) for (16-14) hr, then dilute the broth bacteria by using serial dilution, and then from the nine tube taken 1 ml of broth bacteria and brush on the surface using a cotton swab, the dishes are lefted at room temperature for 30 minutes, then fix disks with silver nanoparticles (which prepared) by sterile forceps, the dishes were incubated at a temperature 37 ° C for 24 hours for bacterial and 7 days for fungal, then measuring the diameter circle inhibition resulting measurement unit.

Characterization of Silver Nanoparticles:

UV-Vis Analysis:

The optical property of AgNPs was determined by UV-Vis spectrophotometer. After the addition of AgNO3 to the plant extract, the spectras were taken in different time intervals up to 24 hr between 350 nm to 500 nm. Then the spectra was taken after 24 hr of AgNO3 addition.

FTIR analysis:

The chemical composition of the synthesized silver nanoparticles was studied by using FTIR spectrometer. The solutions were dried at 75 ^{o}C and the dried powders were characterized in the range 4000-400 cm-1 using KBr pellet method.

XRD Analysis:

The phase variety and grain size of synthesized Silver nanoparticles was determined by X-ray diffraction spectroscopy (Philips PAN analytical). The synthesized silver nanoparticles were studies with CUK radiation at voltage of 30 kV and current of 20 MA with scan rate of 0.030/s. Different phases present in the synthesized samples were determined by X pert high score software with search and match facility.

SEM Analysis:

The morphological features of synthesized silver nanoparticles from neem plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24 hr of the addition of AgNO3 the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV.

TEM Analysis:

The transmission electron microscope (TEM) forms an image by accelerating a beam of electrons that pass through the specimen. In TEM, electrons are accelerated to 100 KeV or higher (up to 1 MeV), projected onto a thin specimen (less than 200 nm) by means of the condenser lens system, and penetrate the sample thickness either undeflected or deflected. The greatest advantages that TEM offers are the high magnification ranging from 50 to 106 and its ability to provide both image and diffraction information from a single sample.

AFM Analysis:

The atomic force microscope (AFM) is one kind of scanning probe microscopes (SPM). SPMs are designed to measure local properties, such as height, friction, magnetism, with a probe. To acquire an image, the SPM raster-

scans the probe over a small area of the sample, measuring the local property simultaneously.

AFMs operate by measuring force between a probe and the sample. Normally, the probe is a sharp tip, which is a 3-6 um tall pyramid with 15-40nm end radius. Though the lateral resolution of AFM is low (~30nm) due to the convolution, the vertical resolution can be up to 0.1nm.

RESULTS AND DISCUSSION:

Nanoparticles are often referred to as particles with a maximum size of 100 nm, and they exhibit unique properties, which are quite different than those of larger particles. New properties of nanoparticles related to variation in specific characteristics like size, shape and distribution have been demonstrated. Among the noble metals, silver (Ag) is the metal of choice for potential applications in the field of biological systems, living organisms and medicine. Synthesis and characterization of AgNPs from plant extracts may act as reducing and capping agents in silver nanoparticles synthesis.

Alphonsea sclerocarpa is a medicinal plant. People are using this plant in their day to day life all around the world. Green synthesis of silver nanoparticles using aqueous leaf extract of *Alphonsea sclerocarpa* with help of 1mM AgNO3 was proved significant in this study. The fresh suspension of *Alphonsea sclerocarpa* was pale yellow color. However, after addition of $AgNO_3$ and stirring, for reduction into Ag ions and kept at room temperature, the emulsion turned to dark brown after 30 min (Fig. 2, A). This confirmed the synthesis of AgNPs in the mixture solution. It is well known that silver nanoparticles exhibit dark brown color in aqueous solution due to excitation of surface Plasmon vibrations in silver nanoparticles.

The color was changed in the cell free extract when challenged with 1mM AgNO3 from pale yellow to dark brown, attained maximum intensity after 30 min with intensity increasing during the period of incubation indicative of the formation of silver nanoparticles. Control (without silver ions) showed no change in color of the cell filtrates when incubated under same conditions. *Shoreatum buggaia* and *Svensonia hyderobadensis* aqueous extract could synthesize silver nanopartcles within 15 min where as *Boswellia ovalifoliolata* took 10 min to synthesize nanoparticles.

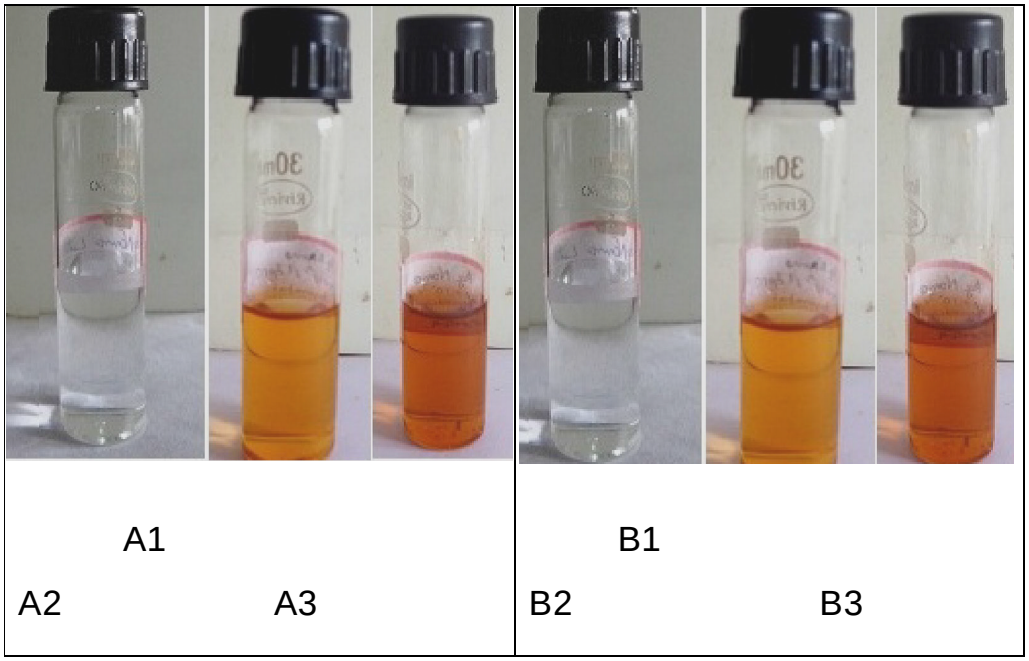

Fig. 2: (A1). Silver Nitrate solution, (A2) *Alphonsea sclerocarpa*leaf aqueous extract, (A3) Silver Nitrate + *Alphonseas clerocarpa* emulsion after 30 min. (B1, B2 and B3) Experimental solutions after 12 hrs.

The important aspect of AgNPs is that their optical properties depend upon the particle size and shape. These optical properties are dominated by the collective oscillation of conduction electrons resulting from the interaction with electro-magnetic radiation [293].

The formation of silver nanoparticles was followed by measuring the surface plasmon resonance (SPR) of the *Alphonsea sclerocarpa* and Silver Nitrate + *Alphonsea sclerocarpa* emulsion at the wavelength range from 300-700 nm. The characteristic silver SPR bands were detected around 400-450 nm (Fig. 4). These absorption bands were assumed to correspond to the silver nanoparticles extra fine and smaller than 25 nm. UV-Vis absorption spectra (Fig. 4) showed that the broad SPR band contained two peaks. These two peaks illustrate the presence of two broad distributions of hydrosol silver nanoparticles.

The reduction of Ag+ ions by combinations of bio molecules found in these extracts (e.g. enzymes/proteins, amino acids, polysaccharides, vitamins etc.) is environmentally benign, yet chemically complex. Due to their exclusive properties, silver nanoparticles (AgNPs) may have several applications, such as catalysts in chemical reactions, electrical batteries and in spectrally selective coatings for absorption of solar energy pharmaceutical components and in chemical sensing and biosensing.

Characterization of silver nanoparticles by UV-Visible spectroscopy:

The reduction of pure silver ions was monitored by measuring the UV-Vis spectrum. The change in colour may be due to excitation of surface Plasmon vibrations of silver nanoparticles (Fig.2,3). The strong absorption observed at (440 nm).

Fig. 3: Picture of flasks containing the *Alphonsea sclerocarpa* leaf extract after incubation in an aqueous solution of AgNO3 solution

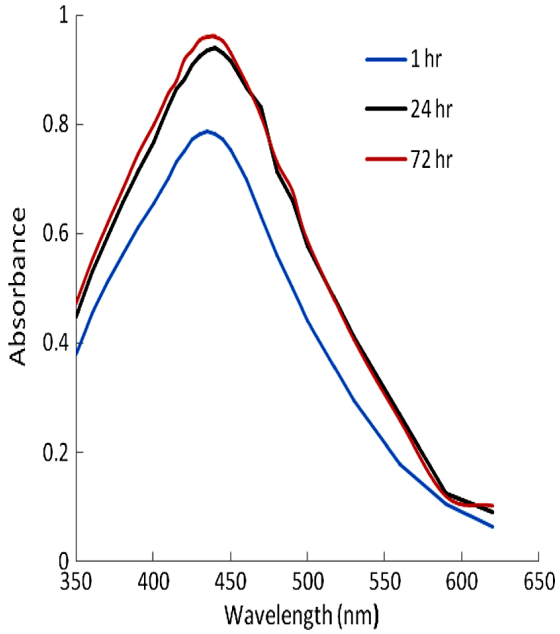

Figure 4: UV-Vis spectrum of silver nanoparticles synthesized using *Alphonsea sclerocarpa* aqueous leaf extract (1,24,72) hr. time interval.

Characterization of silver nanoparticles by FTIR:

The FTIR measurements of the aqueous samples were carried out to identify the possible interactions between silver and bioactive molecules, which may be responsible for the synthesis and stabilization of silver

nanoparticles with the capping agent available in the leaf extract filtrate. The silver nanoparticles are stable and well dispersed. The FTIR spectrum of silver nanoparticles showed three distinct peaks, 462.86, 1643.82 and 3360.72cm−1 (Fig. 5).

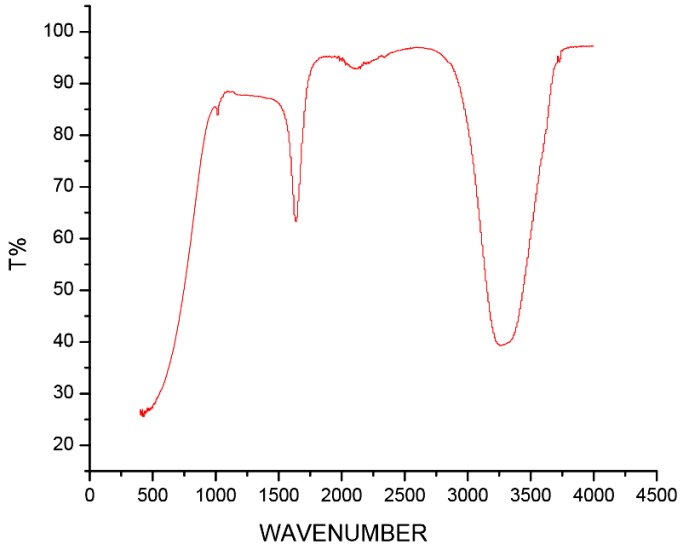

Fig. 5 . FTIR spectrum of silver nanoparticles.

Characterization of silver Nanoparticles by XRD:

The XRD pattern shows four peaks in the whole spectrum of 2 values, ranging from 20 to 80. XRD spectra of pure crystalline silver structures was matched with by the Joint Committee on Powder Diffraction Standards (file nos. 04-0783 and 84-0713). A comparison of our XRD spectrum with standard confirmed that the silver nanoparticles had been formed in the form of nanocrystals. It was evidenced by the peaks at 2 values of 36.25°, 59.37°, 62.60° and 67.62° corresponding to 111, 200, 220 and 311 planes for silver, respectively (Fig.6).

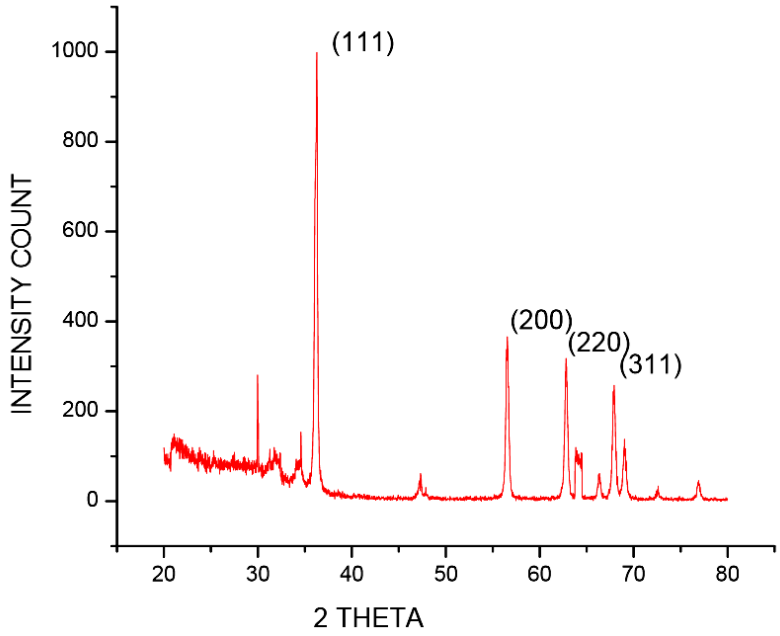

Fig.6. X-ray diffraction pattern of silver nanoparticles

Characterization of silver nanoparticles by SEM:

SEM analysis shows high-density AgNPs synthesized by *Alphonsea sclerocarpa* leaf extract. It was shown that relatively spherical and uniform AgNPs were formed with diameter of (5- 50)nm. The SEM image of silver nanoparticles was due to interactions of hydrogen bond and electrostatic interactions between the bioorganic capping molecules bound to the AgNPs. The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent. The larger silver particles may be due to the aggregation of the smaller ones, due to the SEM measurements (Fig. 7).

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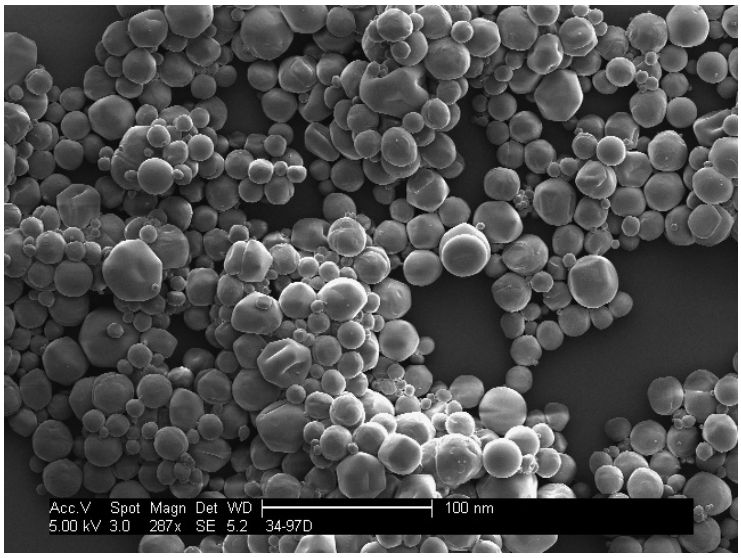

Fig.7 .SEM micrograph showing silver nanoparticles of various size ranges 5-50 nm.

Characterization of silver nanoparticles by TEM:

TEM are most powerful method to determine the morphology and size of nanostructures. TEM micrographs of the synthesized silver nanoparticles confirmed the formation of spherical nanoparticles with a size range of 5-50 nm (Figure 8).

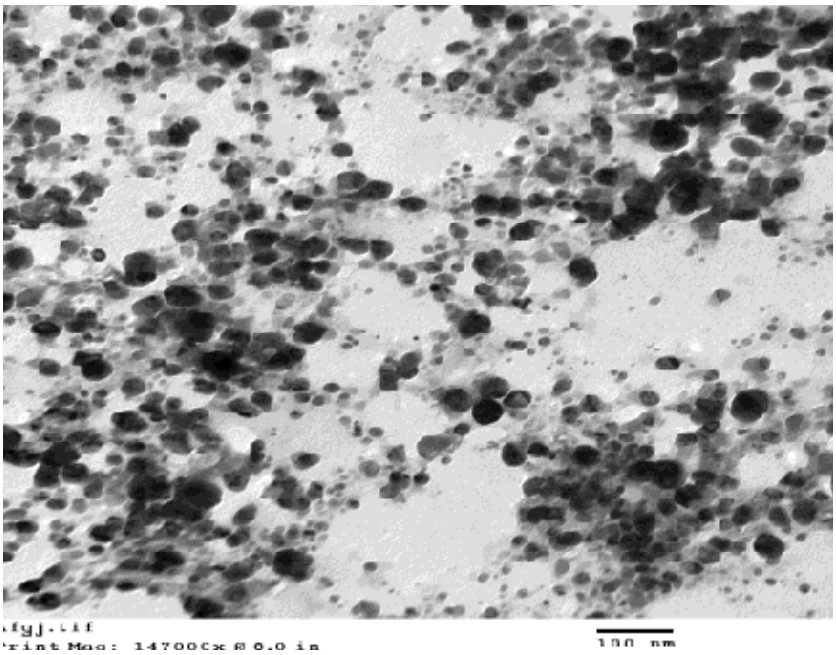

Fig.8.TEM micrograph showing silver nanoparticles of various size ranges 5-50 nm

AFM images of synthesized silver nanoparticles:

The biosynthesized silver nanoparticles were further confirmed by AFM micrographic images (Fig.9 a). It has been reported that the silver nanoparticles size was measured using line profile determination of individual particles in the range of 24.631 nm (Fig.9 b). The fabricated silver nanoparticles were imaged by AFM to understand the exact configuration of the fabricated silver nanoparticles and also used to verify that the silver nanoparticles were more or less homogenous in size and were spherical in shape. The particle size was measured using line profile determination of individual spherical shaped particles in the range of 18-48 nm which was previously confirmed by FE SEM micrographic images. The 3-dimensional structure image of the synthesized AgNPs was shown in (Fig. 9 c).

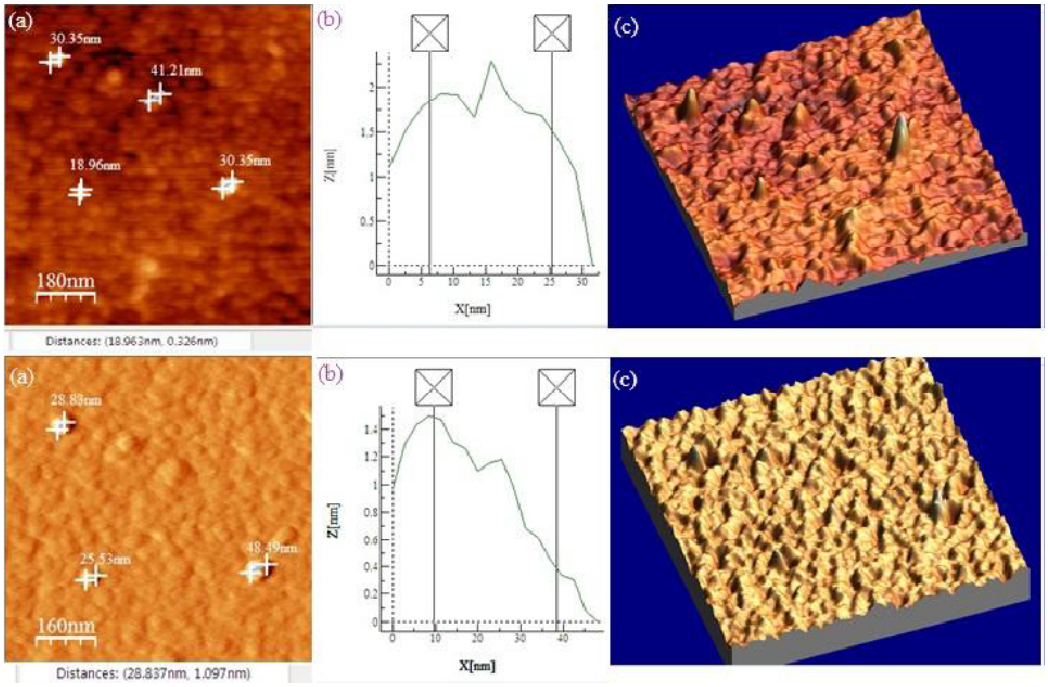

Fig.9 AFM images of synthesized AgNPs showed the spherical shaped particle size of 5-50 nm (a), particle size determination profile of the AFM image (b), the 3- dimensional structure of AgNPs (c).

Determination of antimicrobial activity:

The synthesized silver nanoparticles shows significant antimicrobial activity against bacterial pathogens *Bacillus subtilis, Staphylococcus aureus, Pseudomonas citronellolis, Klebsiella pneumonia, Lactobacillus acidophilus, Enterobacter aerogenes, Candida albicans and Aspergillus niger* (Fig. 10) using agar well diffusion methods. The synthesized silver nanoparticles showed highest antimicrobial activity against gram positive bacteria *Staphylococcus aureus* (MTCC-6908) (Fig.11) showed average zone of inhibition of (26 mm). The Gram negative bacteria *Pseudomonas citronellolis* (MTCC-1191) (Fig.12) showed average zone of inhibition of (18.3 mm). The Fungi *Candida albicans* (MTCC-4748) (Fig.13) showed average zone of inhibition of (22.2 mm). The silver nanoparticles causes an increase in cell

membrane permeability and results finally in cell death [298]. The present study showed a simple, rapid and economical route to synthesized Silver nanoparticles.

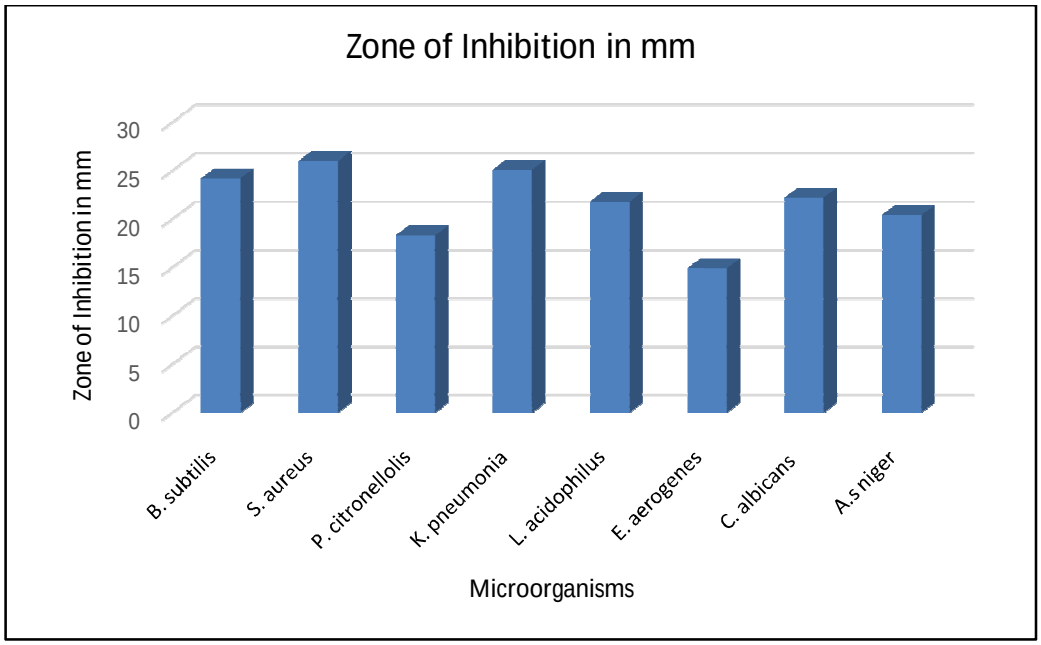

Fig.10. Antimicrobial activities of silver nanoparticles against bacteria and fungi.

All values represented are average of results of three separately conducted experiments.