

Swift Green Synthesis of Silver Nanoparticles using Aqueous Extract of *Tamarindus indica* Leaves and Evaluation of its Antimicrobial Potential

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ABSTRACT: In this report, a simple photo-catalytic rapid green synthesis methodology has been developed for biosynthesis of silver nanoparticles (AgNPs) using aqueous extract *Tamarindus indica* (AET) leaves. The developed process was able to synthesize AgNPs within 5 min without any instrumental support. External reducing agent or stabilizer is not required because AET alone acts as phyto-reducer and capping agent. Rapid change in color of reaction mixture from yellowish green to reddish brown within 5 min in direct sun light was primary indication of AgNPs synthesis. UV-visible spectroscopy confirmed the biosynthesis through SPR band for AgNPs at λ_{max} of 432 nm. The process parameters were manually optimized and then produced AgNPs were systematically characterized using FESEM, EDX, HRTEM, XRD and FTIR techniques. AgNPs were discrete and spherical with average size distribution of 32.4 nm. Particle crystalline in nature and their crystal lattice was of face centered cubic (fcc). Synthesized AgNPs showed good antibacterial potential against both Gram positive and Gram negative bacteria.

KEYWORDS: AgNPs; Antimicrobial activity; Green synthesis; Photo-induced; *Tamarindus indica*

I.INTRODUCTION

Nanomaterials have opened new avenues of solutions for various scientific challenges due to their unique properties [1, 2]. Among these nanomaterials, the nanoparticle (NPs) of noble metals, especially of silver (Ag) and gold (Au) have gained much attention due to their dazzling plasmonic properties, sensing abilities, antibacterial activities and vast biomedical applications [3-7]. Chemical and physical methods such as chemical reduction, electrochemical techniques, sputter deposition and laser ablation are the conventional routes for the synthesis of nanoparticles [8]. The problems associated with these conventional methods are; extensive use of chemicals, expensiveness, aggregation of synthesized nanoparticles, non-ecofriendly nature of the process and requirement of sophisticated instrumentation [9]. Currently, the green route for synthesis of nanomaterials is gaining popularity because of its superiority over the conventional methodologies on above mentioned issues and limitations [9]. Microbial route and herbal route are the two alternative approaches under green synthesis [10, 11]. Although microbial route for nanoparticle synthesis is well reported but it needs specific growth media and aseptic conditions to grow the microbial biomasses thus the process is costly and sophisticated. To avoid these problems, we chose plant extract mediated herbal route for the green synthesis of silver nanoparticles (AgNPs) because process was simple, economical and eco-friendly.

Tamarindus indica is a member of *Fabaceae* family and commonly known as 'Imli'. This is a very common fruit plant distributed throughout various regions of India. Therefore, its leaves can be an inexpensive and easily available source of material for nanoparticle synthesis. Present study deals with a photo-induced and phyto-mediated green approach for the

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swift biosynthesis of silver nanoparticles (AgNPs) using aqueous extract of *Tamarindus indica* leaves (AET) for both as a bioreductant as well as capping agent. The process was optimized and synthesized AgNPs were characterized through UV-Visible spectroscopy, FTIR, XRD, FESEM, EDAX and HRTEM analysis. Thereafter, the antibacterial efficacies of AgNPs were examined for their antimicrobial efficacy against both the Gram positive and Gram negative bacteria.

II. MATERIALS AND METHOD

2.1 CHEMICALS

For biosynthesis of AgNPs, silver nitrate (AgNO_3) was used as precursor and was procured from Merck (Mumbai, India). Nutrient Agar (NA) media was utilized for maintaining the bacterial cultures whereas Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) media were used for the assessment of antimicrobial efficacy of AgNPs. All the ingredients of NA and MHA and MHB (complete media) were sourced from Hi-Media (Mumbai, India). All chemicals utilized in the study were of analytical grade and utilized without any additional purification. Double distilled deionized water was used to prepare all the aqueous solutions and media. Fresh leaves of *Tamarindus indica* were collected from campus area of Indian Institute of Technology (Banaras Hindu University), Varanasi, India.

2.2. AQUEOUS EXTRACT PREPARATION FROM *Tamarindus indica* LEAVES

The collected leaves of the *Tamarindus indica* were repeatedly and thoroughly washed with deionized water and then air dried under shade condition for 3 h at room temperature to remove excess water. 20.0 g of pre-washed leaves were chopped in to fine pieces and boiled for 5 min in 100 mL of distilled water under constant stirring on a hot plate magnetic stirrer. After cooling, the boiled leaves were crushed and extracted through muslin cloth. Obtained extract was further filtrated through 'Whatman filter paper No 1' and collected. Prepared extract was stored as stock solution at 4°C and used within 3days.

2.3. BIOSYNTHESIS OF SILVER NANOPARTICLES (AgNPs)

AgNPs biosynthesis experiments were started with two sets of reaction mixtures each having 10 mL of 1.0 mM AgNO_3 solution and 2.5% (v/v) AET inoculum dose. The first set of reaction mixture was kept in direct sun light exposed condition while other set was kept in dark condition in incubator at same temperature as for first set in ambient environment (36°C, pH 7.0). Biosynthesis of AgNPs was confirmed by color change of reaction mixture and screening the samples from both the reaction mixtures through UV-visible spectrophotometer (Evolution 201, Thermo Scientific) for presence of characteristic surface plasmon resonance (SPR) band of AgNPs. After verification of the photocatalytic action of solar light on the process, further all the biosynthesis experiments were conducted in direct sunlight in order to optimize the process parameters using one factor at a time approach. The exposure time was optimized through scanning the samples at different exposure times (0 to 45 min). Effect of AET inoculum volume and AgNO_3 concentration was also investigated by varying inoculum volume from 2.5% to 12.5% (v/v) and AgNO_3 from 1.0 mM to 6.0 mM. After process optimization, Ag-NPs were produced in bulk (500 mL) at optimum conditions. The reaction solution was centrifuged at 15,000 rpm for 10 min, supernatant was discarded and pellet was resuspended in to double distilled deionised water. Same step was reperformed with acetone. The process was repeated four times and final mass of Ag-NPs was collected after lyophilization of aqueous suspension.

2.4. CHARACTERIZATION OF SYNTHESIZED AgNPs

The primary characterization was conducted through screening the characteristic optical properties (SPR band) of AgNPs in the reaction mixture through UV-Visible spectroscopy (Evolution 201, Thermo Scientific) in the range of 300 to 700 nm. Morphology of synthesized AgNPs was determined by Field Emission Scanning Electron Microscopy (FESEM)

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Hitachi H-7100) at accelerating voltage of 10 kV, beam current 1 nA. The presence of metallic silver in the sample was determined by Energy Dispersive X-Ray Spectroscopy (EDX) which was equipped with FESEM. Further, detailed morphology of the AgNPs was explored through High Resolution Transmission Electron Microscopy (HRTEM) (TECNAI 20 G2-electron microscope) operated at accelerating voltage 200 kV. To analyze the crystal lattice, X-ray diffraction analysis was also conducted through X-ray diffractometer (Rigaku Miniflex II) having Cu K α radiation source and Ni filter to analyze the crystalline structure of AgNPs. To investigate the involved functional groups of phytochemicals in biosynthesis and stabilization of AgNPs, the Fourier Transform Infrared (FTIR) spectroscopy of AgNPs as well as of AET was carried out in the range of 4000–400 cm⁻¹ using Varian 3100 FTIR spectrophotometer (Perkin Elmer Spectrum 100).

2.5. ANTIMICROBIAL EFFICACY OF AgNPs

2.5.1. MICROORGANISMS AND INOCULUM PREPARATION

The antimicrobial activity of synthesized AgNPs was evaluated against both Gram positive bacteria *Staphylococcus aureus* (*S. aureus*, MTCC 9886) and Gram negative bacteria *Eischherichia coli* (*E. coli*, MTCC 1680). Both the bacterial test strains were acquired from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India and were preserved on NA slants at 4°C. Inoculums were prepared in 25 mL of MHB by suspending a single bacterial colony of each test bacteria in separate broth, picked from NA slant with a sterile loop. Inoculums were incubated overnight at 37°C on 100 rpm shaking speed. The optical densities of the resultant microbial inoculums were adjusted at equivalent to the 0.5 McFarland standard (0.5 mL of 0.048 M BaCl₂ in 99.5 mL of 0.18M H₂SO₄) by adding sterile MHB broth. Each inoculum thus prepared would contain bacterial number approximately 10⁷ cfu/ml [12].

2.5.2. DISC DIFFUSION ASSAY

The disc diffusion assay was conducted to evaluate the antimicrobial activity of the biologically synthesized AgNPs against both *S. aureus* and *E. coli*. Assay was performed as per guideline of National Committee for Clinical Laboratory Standards [13]. Prepared bacterial inoculums (10⁷ cfu/ml) were spread plated over MHA plates. 6 mm sterile filter disc was first soaked with 20 μ L AgNPs solution of 50 ppm concentration and then gently placed on to the surface of MHA plates. Two discs separately inoculated with 20 μ L of AET, AgNO₃ (5 mM) and were also included in experiment for compare. A control disc containing buffer solution was also applied to the plate along with test discs. The inoculated plates were incubated at 37°C for 24 h and zone of inhibitions were measured.

2.5.3. ASSAY FOR MINIMUM INHIBITORY CONCENTRATION OF AgNPs

The minimal inhibitory concentration (MIC) of AgNPs against both *E. coli* and *S. aureus* were also determined by an amended broth macro-dilution method [14]. For MIC determination, stock solutions of 40 different concentrations of AgNPs ranging from 1.0 μ g/mL to 40.0 μ g/mL were prepared in autoclaved MHB broth. Sterile test tubes containing 2.0 mL of test bacterial cultures (10⁷ cfu/ml) were arranged in two sets (one set for *E. coli* and another for *S. aureus*) for each bacterial strain to cover the 40 different concentrations of AgNPs in duplicate. Thereafter, each test tube was individually supplemented with 2.0 mL of each concentration of AgNPs accordingly, thus the final volume of media in the test tubes become 4.0 mL. This results in a 1:2 dilution of both AgNPs concentrations and bacterial cultures (0.5–20.0 μ g/mL and 5 \times 10⁶ cfu/ml respectively). Pure bacterial inoculum in MHB was taken as positive control whereas MHB with AgNPs but without bacterial inoculum was taken as and negative control. All test tubes were incubated at 35°C for 24 h. After incubation, microbial growth was estimated by measuring the optical density (O.D) at 600 nm through U.V-Visible spectrophotometer (Evolution 201, Thermo Scientific). MIC endpoint was concluded as the lowest concentration of AgNPs showing no visible growth after 24 h incubation.

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III.RESULTS AND DISCUSSION

3.1. BIOSYNTHESIS AND PHOTOCATALYTIC VERIFICATION

Experiments were started with tow set of reaction mixtures each having 10 mL of 1.0 mM AgNO₃ solution and 2.5% (v/v) AET inoculum dose (arbitrarily selected). one set was kept in direct sun light while other in dark condition. The reaction mixtures kept in sun light exhibits rapid color change from light pale-green to dark reddish brown within 5 min after inoculation of AET into AgNO₃ solution (Fig. 1). However, second set of reaction mixture, which was kept at dark condition, did not showed rapid color change and fails to attain the same degree of color change even after 4 h of reaction time. The appearance of reddish brown color in reaction mixture, clearly indicate the biosynthesis of AgNPs. Color appears in the reaction mixture was due to surface plasmon resonance of AgNPs [15]. This huge time gap for appearance of color in both conditions clearly indicates that solar light had a positive impact on AgNPs biosynthesis i.e. process is photo catalytic in nature.

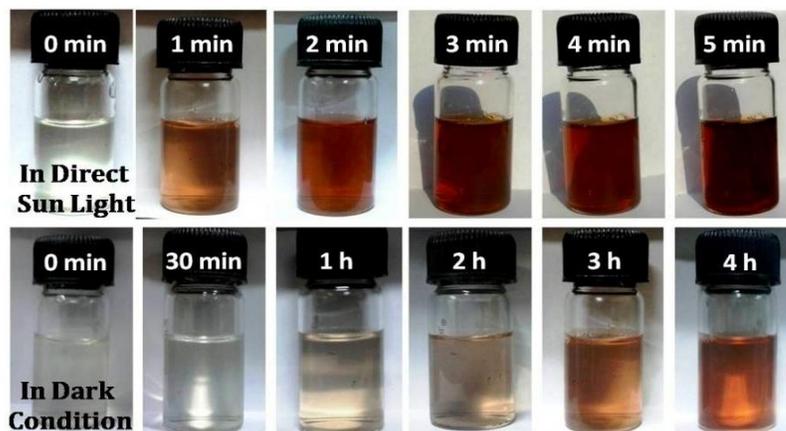


Fig 1: Color variation of reaction mixture with proceeding of time in bright sunlight and in dark conditions.

Biosynthesis of AgNPs was further confirmed by screening the presence of characteristic SPR band in samples of reaction mixtures through UV-visible spectroscopy. Samples withdrawn after 5 min from first set (sun light exposed) of reaction mixture showed a sharp SPR band at 432 nm which is characteristic of AgNPs, whereas sample taken from second set after 5 min illustrate a very weak SPR band (Fig. 2a). This difference in SPR band intensity further confirms the photo-catalytic nature of current process therefore further all the experiments were carried out in direct sun light condition.

3.2. EFFECT OF REACTION TIME ON BIOSYNTHESIS OF AGNPS IN DIRECT SUNLIGHT

Effect of reaction time was investigated on the biosynthesis of AgNPs by keeping the reaction mixture in direct sunlight (at above mentioned conditions) under consistent screening of samples through UV-visible spectroscopy. Samples were withdrawn at regular time interval of 5 min. Results are shown in Fig. 2b. The state of equilibrium was confirmed by monitoring the increment in SPR band intensity. The continued increase in the SPR peak intensities of AgNPs up to 40 min indicate that the reduction of silver ions is in process whereas no significant improvement in peak intensity after 40 min signified the establishment of equilibrium. On the basis of these results, 40 min exposure time was fixed for further experiments.

3.3. EFFECT OF AgNO_3 CONCENTRATION

After reaction time optimization, the effect AgNO_3 concentration on the biosynthesis of AgNPs was investigated in the range of 1.0 mM to 6.0 mM. For optimization of AgNO_3 concentration; other parameters were kept constant at 2.5% AET inoculum dose and 40 min of sun light exposure time. SPR bands for AgNPs at different AgNO_3 concentrations are represented in Fig. 2c. Color of reaction mixture was observed to be varied for different AgNO_3 concentration for the fixed reaction time. Many previous scientific reports have pointed out the fact that the color of reaction mixture depends on size of nanoparticles [16]. Thus, it is clear that AgNO_3 concentration affect the particle size distribution for in the reaction mixture for current biosynthetic system. Initial increase in AgNO_3 concentration from 1 mM to 5 mM results sharper and more intense SPR bands with regular blue shift in λ_{max} (432 nm to 422 nm) whereas further increase in AgNO_3 concentration from 5 mM to 6 mM results broader and less intense SPR bands. Increase in the band intensity indicates the increase in number of synthesized AgNPs per unit volume of reaction mixture whereas blue shift signifies the reduction in the size of AgNPs. Moreover, broadening and decrement in intensity of SPR band at higher AgNO_3 concentration (>5 mM) indicated the synthesis of larger AgNPs in lesser number. Therefore, 5mM AgNO_3 concentration was selected as optimum for current biosynthetic process.

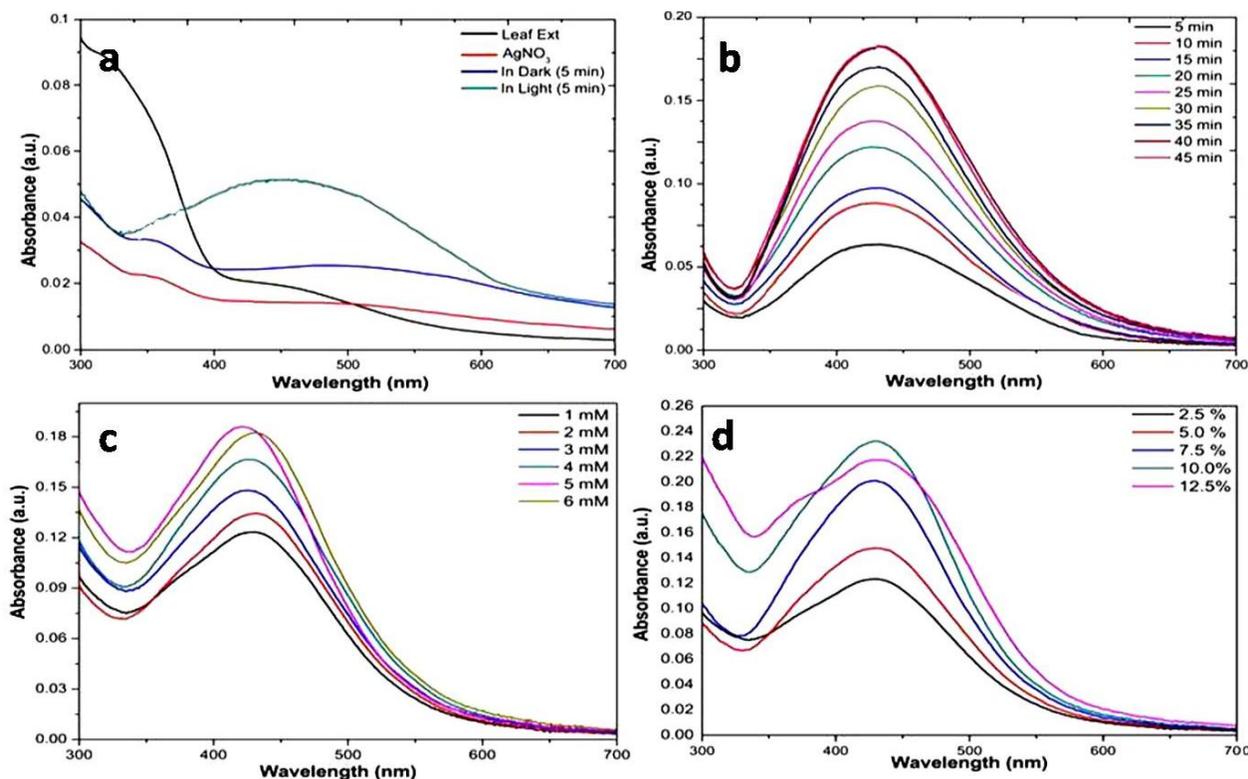


Fig. 2: (a) UV-Visible spectra showing effect of photo-induction on synthesis of AgNPs via AET. (b) UV-Visible spectra showing effect of time on AgNPs synthesis (conditions; AgNO_3 conc. 1 mM, AET inoculum dose 2.5% (v/v)). (c) UV-Visible spectra showing effect AgNO_3 conc. on synthesis of AgNPs. (conditions; reaction time of 40 min in sunlight and 2.5% (v/v) AET inoculum dose). (d) UV-Visible spectra showing effect AET inoculum dose on synthesis of AgNPs. (conditions; AgNO_3 concentration; 5mM and reaction time of 30 min in sunlight).

3.4. EFFECT OF “AET” INOCULUM DOSE

The quantity of AET inoculum in reaction mixture was optimized as last process variable. For optimization; AET inoculum dose (v/v) was varied from 2.5% to 12.5% whereas other parameters were kept constant at 5 mM AgNO₃ concentration and 40 min of reaction time. The results are shown in Fig. 2d. It was observed that the intensity of SPR bands increased up to 10.0% (v/v) of AET inoculum dose; indicating increased synthesis of AgNPs with increase in inoculum dose [17]. Further increment in inoculum dose from 10.5%, to 12.5% (v/v), the intensity of single SPR band decreased and band became broader which indicated that the number of biosynthesized AgNPs decreased at very high inoculum dose. A minor shift in wavelength towards red region (from λ_{max} 432 nm to 438 nm) was also observed on increasing inoculum dose indicate the increase in size of synthesized AgNPs at higher inoculum dose .

3.5. CHARACTERIZATION AND STABILITY OF AgNPs

After process optimization, AgNPs were synthesized in bulk at optimized process parameters, collected and then characterized through SEM, EDX, TEM, XRD, and FTIR, analysis.

FESEM analysis was performed after drop coating of sample over the thin sheet of the aluminium. SEM images (Fig 3a) confirmed the synthesis and discrete distribution spherical shaped AgNPs. Energy dispersive X-ray detector (EDX) examination revealed the purity and elemental composition of synthesized AgNPs and it represent a prominent spectral signal of metallic silver around 3 keV (Fig. 3b). Along with elemental signal of silver; the additional signals for oxygen and carbon were also recorded in EDX spectra, which may result due to the presence of integrated biological compounds with nanoparticles. Peak of aluminium was due to aluminium plate over which the sample was coated. Silica peak may result via ‘Si’ impurity in ‘Al’ sheet or probable occurrence of some silicious compounds in AET.

HRTEM analysis was also conducted to explore detailed morphological characteristics of synthesized AgNPs. The HRTEM image (Fig 3c) revealed that most of the AgNPs were roughly spherical in shape. Size distribution histogram (Fig. 3d) of AgNPs corresponding to HRTEM image represented that majority of nanoparticles were in range of 15 nm to 55 nm having average size distribution of 32.74 nm.

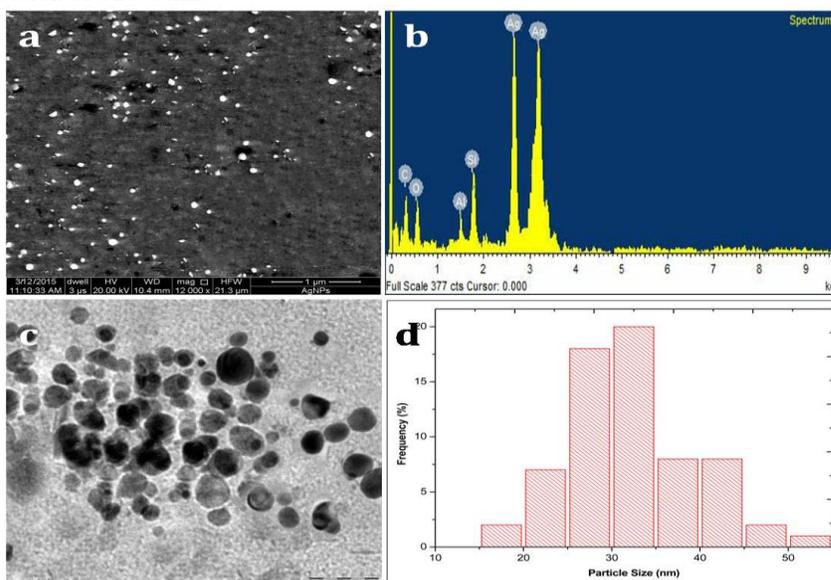


Fig. 3. (a) FESEM images of AgNPs; synthesized via AET at optimum process parameters (b) EDX spectrum of AgNPs of corresponding FESEM images sample (c) HRTEM images of AgNPs (d) AgNPs Histogram.

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Fig. 4a showed the XRD spectra of synthesized AgNPs which represent following diffraction peaks at $2\theta = 38.46^\circ$, 44.17° , 64.72° and 77.67° . These peaks attributed to the (111), (200), (220) and (311) Bragg reflections indicating face centered cubic (fcc) crystal lattice of AgNPs.

FTIR analysis of both AET and AgNPs (Fig. 4b) was also conducted to investigate the possible functional groups of biomolecules involved in synthesis and stabilization of AgNPs. IR spectra of AET showed prominent bands and peaks at 1626.45 , 1446.63 , 1371.76 and 1035.85 cm^{-1} . It also poses a broad band around $3200\text{-}3400$ cm^{-1} which correspond to ‘-OH’ groups of tannin, flavanoides and other phenolic compounds. This broad band ($3200\text{-}3400$ cm^{-1}) also corresponds to ‘-OH’ groups of glucose and ‘-NH’ stretching of the proteins [17, 18, 19]. Peak at 1626.45 cm^{-1} assigned to primary and secondary amines as well as amide linkage of proteins [19, 20]. The absorbance peak around 1446.63 cm^{-1} attributed to N-H stretching, O-H deformation and $-\text{CH}_2$ scissoring [21]. Sharp band around 1371.76 & 1035.85 cm^{-1} denotes stretching vibrations carboxylic groups and ‘-CN’ stretching of proteins respectively [17-20]. IR spectra of AgNPs showed shifting in following peaks and bands as compared with IR spectra of AET: peak at 1626.45 sifted to 1651.62 and 1446.63 to 1404.32 cm^{-1} . An intense narrowing in the broad band ($3200\text{-}3400$ cm^{-1}) was also observed. These siftings in peaks and narrowing of band indicated the involvement of mainly hydroxyl, amino and amide groups of phyto-chemicals present in AET for the biosynthesis of AgNPs. These groups can majorly be contributed by phenolic compounds (tannins, terpenoides flavanoids) and proteins present in AET [22].

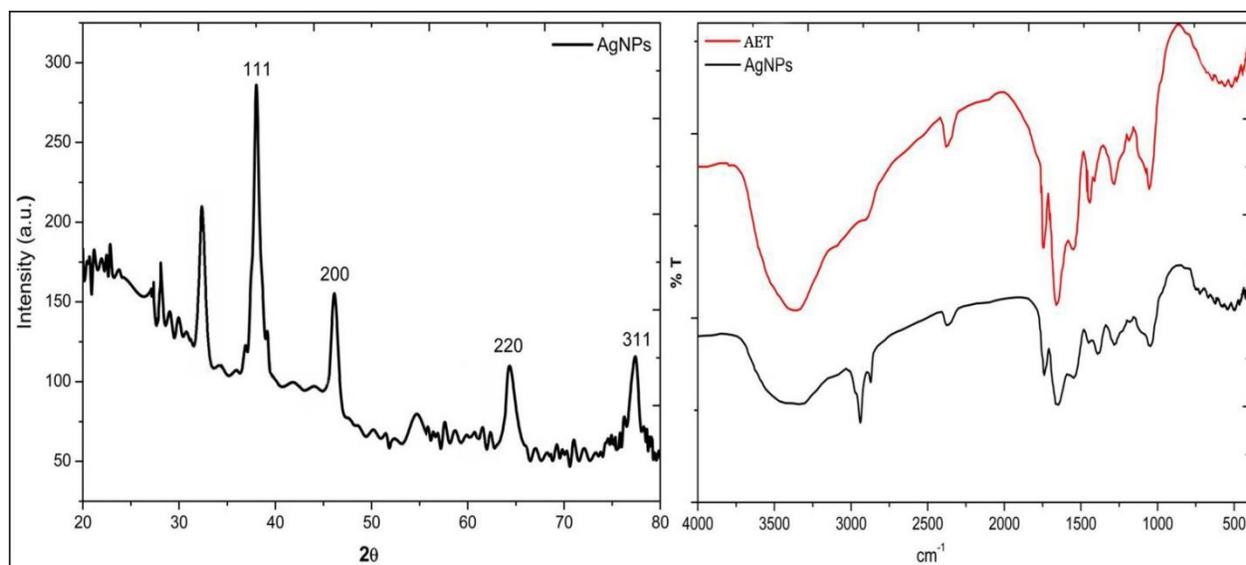


Fig. 4. (a) X-ray diffraction pattern of synthesized AgNPs. **(b)** FTIR spectra of the AET and AgNPs.

3.6. DISC DIFFUSION ASSAY AND MINIMUM INHIBITORY CONCENTRATION (MIC)

Disc diffusion assay was performed for evaluating the antibacterial efficacy of AgNPs against *E. coli* and *Staphylococcus aureus*. Results for disc diffusion assay are shown in Fig 5a & 5b, which represents zone of inhibitions (ZOIs) around individual discs, inoculated with $20\mu\text{L}$ suspensions of AgNPs (50 ppm), AET and 5 mM AgNO_3 solution and control buffer solution.

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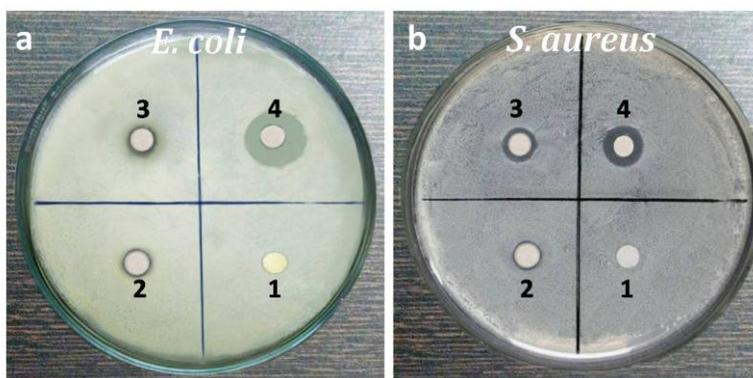


Fig. 5: (a) Antimicrobial efficacy of synthesized AgNPs against; (a) *E. coli* (Gram -ve) (b) *Staphylococcus aureus* (Gram +ve). Disc (1) Buffer solution, (2) AET (3) AgNO₃ and disc (4) AgNPs.

AgNPs created 18 mm and 14 mm wider ZOI on *E. coli* and *Staphylococcus aureus* test plates respectively. Results had proven the significant antibacterial effects of synthesized AgNPs on tested bacteria of both gram classes. It is also clear from the results that AgNPs were more effective against *E. coli* (Gram negative) as compare to *Staphylococcus aureus* (Gram positive). Because the Gram negative bacteria poses weaker cell wall due to less peptidoglycan content as compare to Gram positive bacterial cell wall therefore showed greater susceptibility for AgNPs [23]. These findings are in agreement with many previous reports [24-26]. Minor zone of inhibitions were also observed around the discs containing AET and AgNO₃. Interaction of free silver ions of AgNO₃ with vital enzymes of bacteria provides a mild antibacterial activity to AgNO₃ whereas herbal extracts poses antimicrobial activity due their phytochemicals. AgNPs exhibits antimicrobial activity due to its ability to disrupt cell wall; produce Reactive Oxygen Species (ROS) mediated toxicity and interfering activity with DNA replication [27].

MIC determination for AgNPs was also done though macro-dilution method against both bacterial strains after primary confirmation of antimicrobial activity of AgNPs through disc diffusion assay. MIC for *E. coli* and *S. aureus* was found to be 16.50 µg/mL and 19.5 µg/mL. Because MIC value for *E. coli* is low; this clearly indicates the better susceptibility of *E. coli* for AgNPs as compare to *S. aureus*. MIC values further support the results of disc diffusion assay.

IV.CONCLUSION

Present study demonstrated a very simple green chemistry approach for the synthesis of silver nanoparticles at ambient conditions by using *Tamarindus indicaleaf* extract (AET). Developed process was photocatalytic, swift, economic and eco-friendly in nature for the large scale production of silver nanoparticles. Presented methodology required no instrumental support or additional energy supply like heat and stirring. AET alone was capable to provide both the bioreductant and stabilizer required for the nanoparticle production. The formation of AgNPs was observed by change in color of reaction mixture from light green to dark reddish brown. Further the biosynthesis of AgNPs was confirmed by UV-vis spectroscopy. The process was manually optimized and thereafter the synthesized AgNPs were systematically characterized through FESEM, EDX, HRTEM, XRD and FTIR. 40 min of reaction time, 5.0 mM AgNO₃ concentration and 10.0 % (v/v) of AET inoculum dose was found to be optimal for the process. Synthesized AgNPs were spherical in shape, crystalline in nature having face centered cubic (fcc) crystal lattice. The average particle size of synthesized AgNPs was found to be 32.74 nm. Antimicrobial studies showed that AgNPs were

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more effective against Gram negative bacteria as compare to Gram positive bacteria. Thus the current methodology can provide a better alternative over conventional physico-chemical methods for AgNPs synthesis.

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