SECTION II - CHAPTER 5

5.1 Chapter Introduction

In 'Chapter 4', it has been mentioned that positive activity for AgNPs synthesis was found in two plants i.e. *Salvinia molesta* and *Tamarindous indica*. After detailed study on *Salvinia molesta* the potential of *Tamarindous indica* plant was also explored for the synthesis of AgNPs, so that the AgNPs with better antimicrobial activity could be selected on comparative basis. This chapter presents the work on AgNPs synthesis from aqueous extract of *Tamarindus indica* (AET) leaves.

5.2 About *Tamarindus indica*

Tamarindus indica is a member of *Fabaceae* family and commonly known as 'Imli'. This is a very common fruit plant indigenous to tropical Africa and also distributed throughout various regions of India. The genus *Tamarindus* is a monotypic taxon i.e. has only a single species in whole texon. Apart from producing edible fruits the tamarind also used in traditional medicine and metal polishing. The wood is useful in carpentry. It is cultivated all around the world in tropical and subtropical zones. Therefore, its leaves can be an inexpensive and easily available source of material for nanoparticle synthesis. *Fig 5.1* represents the leaves and flowers of tamarind plant.

Fig. 5.1 *Tamarindus indica* leaves

5.3 Materials

All the materials utilized were similar as utilized for AgNPs synthesis through *Salvinia molesta* descripted in 'Chapter 4'.

5.4 Methods

5.4.1 Preparation of aqueous extract of *tamarindus indica* leaves (AET)

Fresh leaves of *Tamarindus indica* were collected from campus area of Indian Institute of Technology (Banaras Hindu University), Varanasi, India. The collected leaves of the *Tamarindus indica* were repeatedly 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. After cooling, the boiled leaves were separated through muslin cloth. Obtained extract was further filtrated through 'Whatman filter paper No 1' and obtained AET was collected**.** Prepared extract was stored as stock solution at 4°C and used within 3days.

5.4.2 Procedure of biosynthesis, characterization and evaluation of antimicrobial efficacy of AgNPs

All the methods of synthesis, characterization and evaluation of antimicrobial potential through disc diffusion method and cell viability test are similar as per descripted in Chapter 4 for *Salvinia molesta*.

5.5 Results and discussion

5.5.1 Primary verification AgNPs Biosynthesis

Experiments were started with tow set of reaction mixtures each having 10 mL of 1.0 mM $AgNO_3$ 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 $AgNO_3$ solution

(*Fig. 5.2*). 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. Reaction was observed till 12h and it was found that although the reaction mixture does not exhibited color change equivalent to light condition but after 12 h the color of reaction mixture was darker than that in case of *Salvinia molesta*. 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 [Mulvaney et al., 1996]. 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.

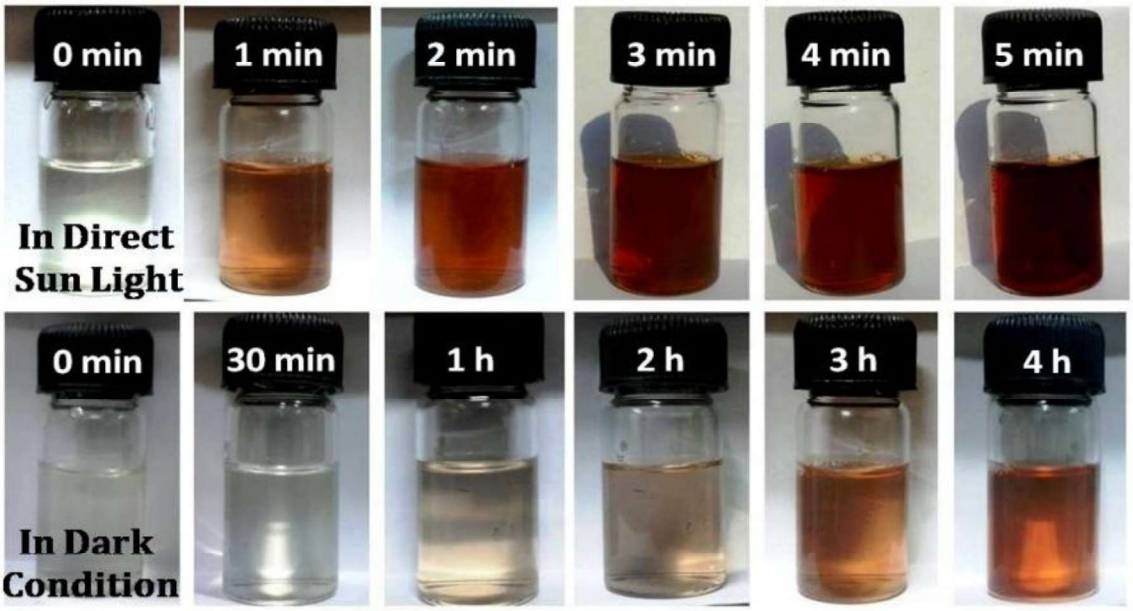

Fig 5.2: Color variation of reaction mixture with proceeding of time in bright sunlight and in dark conditions

Biosynthesis of AgNPs was further confirmed by screening the presence of characteristic SPR of AgNPs 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. 5.3*). 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.

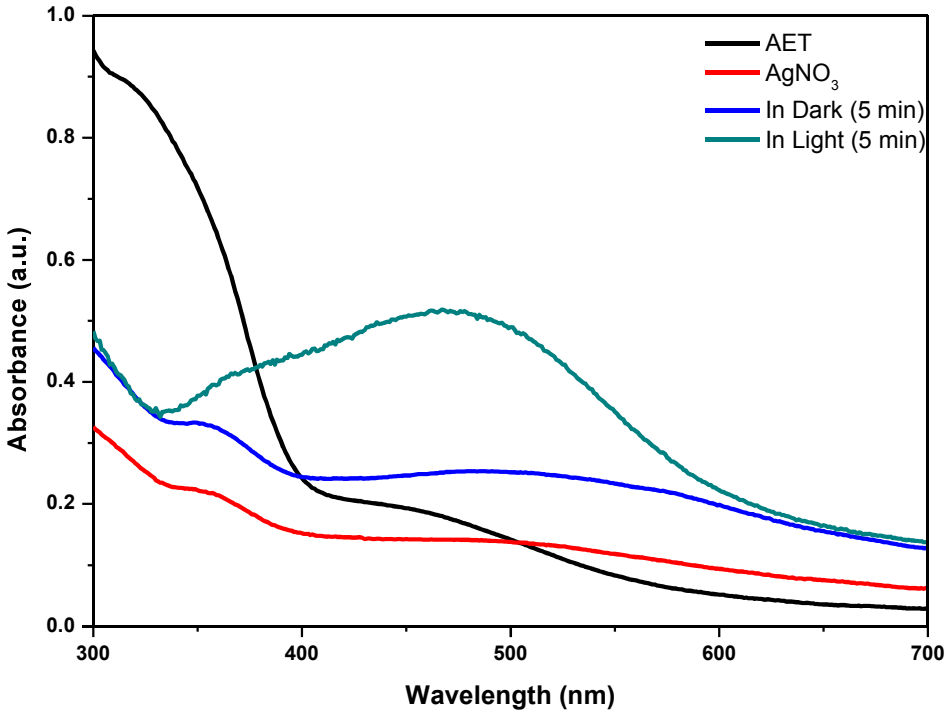

Fig 5.3 UV–Visible spectra showing effect of photo-induction on synthesis of AgNPs using AET

5.5.2 Effect of reaction time on biosynthesis of AgNPs in direct sunlight

Effect of reaction time was investigated and optimized for the biosynthesis of AgNPs by keeping the reaction mixture in direct sunlight under consistent screening of samples through UV-visible spectroscopy. Samples were withdrawn at regular time interval of 5 min. Results are shown in *Fig. 5.4.* The state of equilibrium was confirmed by monitoring the increment in SPR band intensity. Although, the colour change in the reaction mixture was not observed after 5 min, but UV-visible spectroscopy indicated that reaction does not reached at equilibrium in 5 min. The

continued increase in the SPR peak intensities of AgNPs up to 40 min indicate that the reduction of silver ions was in process up to 40 min whereas no significant improvement in peak intensity after 40 min which signified the establishment of equilibrium. On the basis of these results, 40 min exposure time was fixed for further experiments.

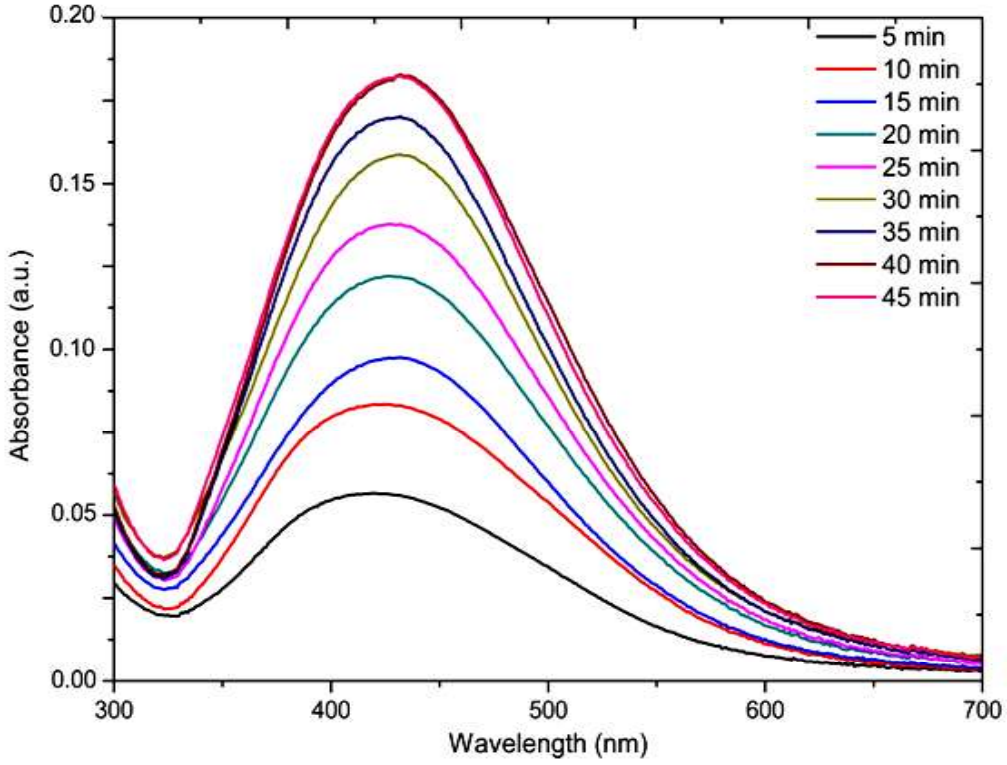

Fig. 5.4 UV–Visible spectra showing effect of time on AgNPs synthesis (conditions; $AgNO_3$ conc. 1 mM, AET inoculum dose 2.5% (v/v))

5.5.3 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. 5.5*. Color of reaction mixture was observed to be varied for different $AgNO_3$ concentrations 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 [Mock et al., 2002]. 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 sift 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 sift signifies the reduction in the size of AgNPs. Moreover, broadening and decrease 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.

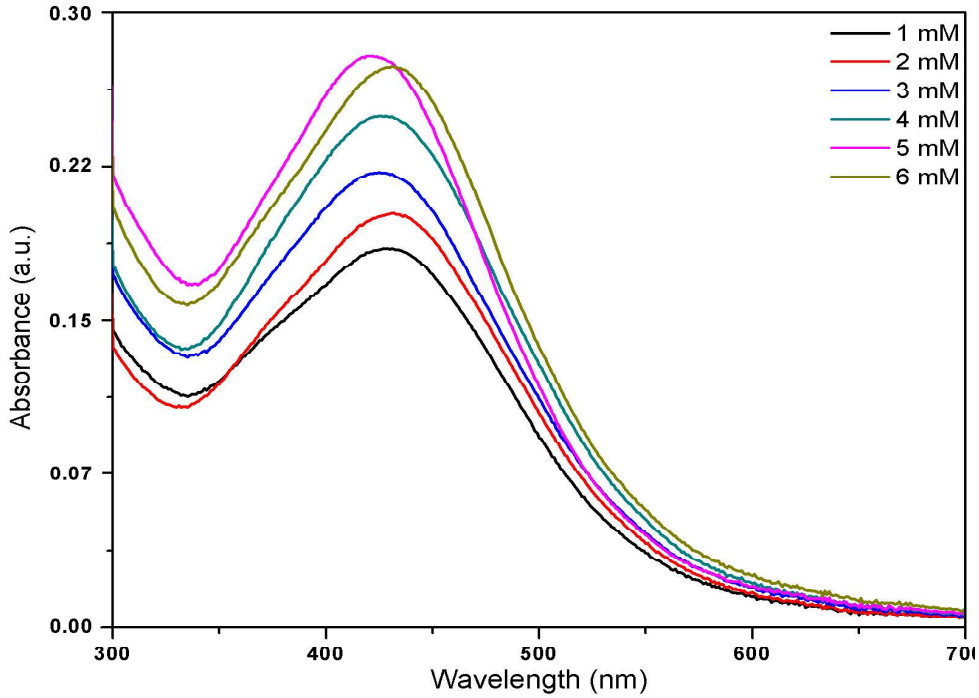

Fig. 5.5 UV–Visible spectra showing effect $AgNO_3$ conc. on synthesis of AgNPs. (conditions; reaction time of 40 min in sunlight and 2.5% (v/v) AET inoculum dose)

5.5.4 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 $AgNO_3$ concentration and 40 min of reaction time. The results are shown in *Fig. 5.6*. 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 [Bai, S., Abraham, 2002]. Further increment in inoculum dose from 10.0%, 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 sift 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.

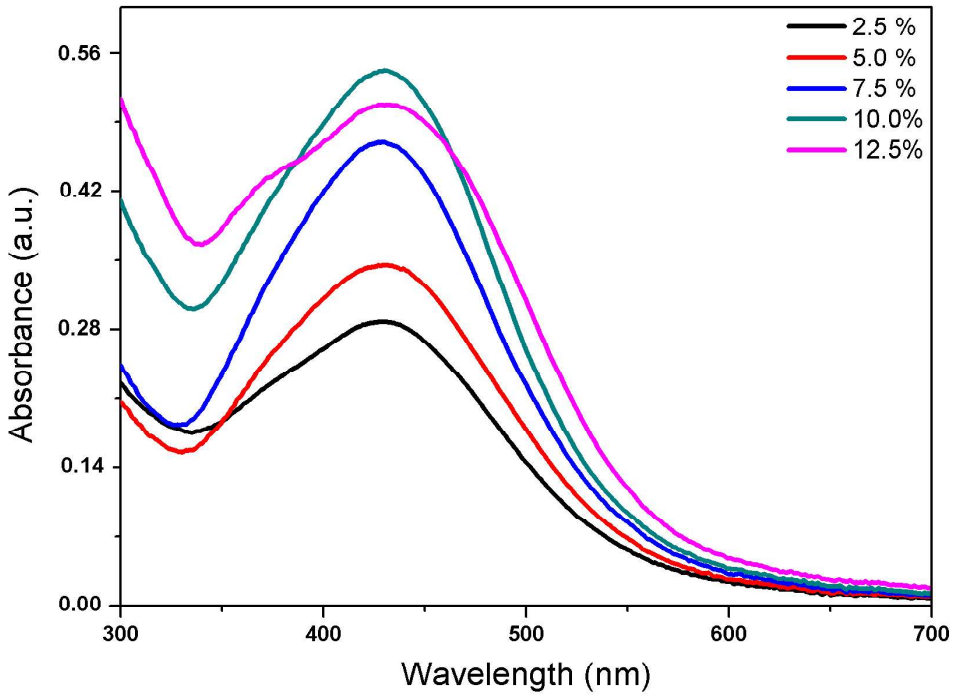

Fig. 5.6 UV–Visible spectra showing effect AET inoculum dose on synthesis of AgNPs. (conditions; $AgNO_3$ concentration; 5mM and reaction time of 30 min in sunlight).

5.5.5 Characterization of AgNPs

After process optimization, AgNPs were synthesized at optimized process parameters, collected and then characterized through FESEM, EDX, HRTEM, XRD, and FTIR, analysis. FESEM analysis was performed after drop coating of sample over the thin sheet of the aluminium. FESEM images (*Fig 5.7 a, b,)* 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. 5.8 a, b). Along with elemental signal of siver; 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 selicious compounds in AET.

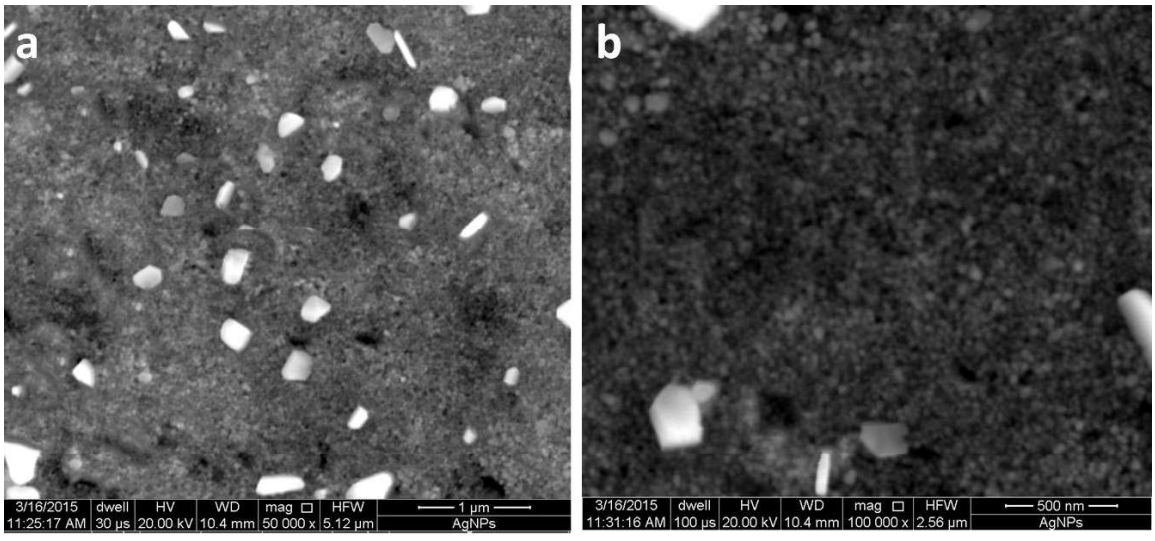

Fig. 5.7 (a,b) FESEM images of AgNPs; synthesized via AET at optimum process parameters

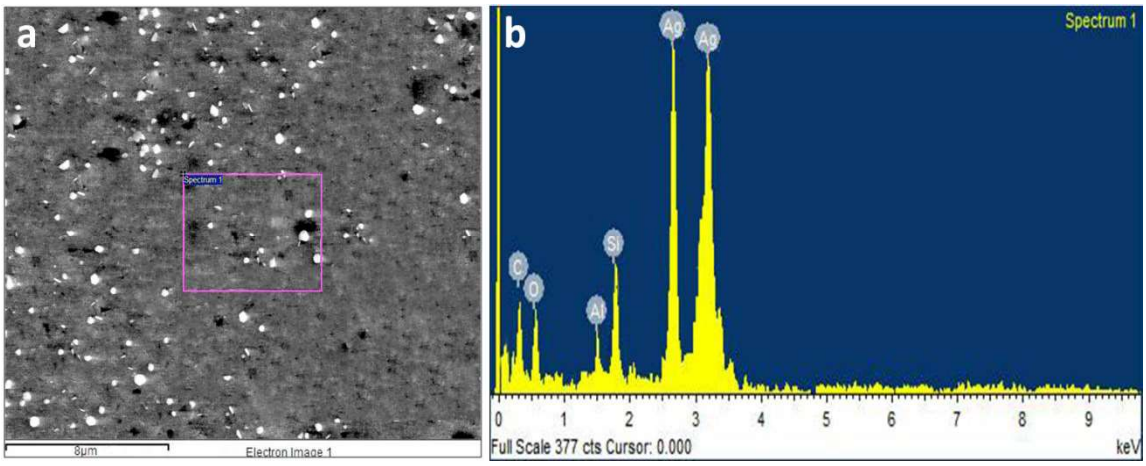

Fig. 5.8 (a) FESEM image of AET synthesized AgNPs and (b) EDX spectrum of AgNPs of corresponding FESEM image

HRTEM analysis was also conducted to explore detailed morphological characteristics of synthesized AgNPs. The HRTEM image (*Fig 5.8 a*) revealed that most of the AgNPs were roughly spherical in shape. Size distribution histogram (*Fig 5.9 b*) 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. Image was processed through 'Image J' software for the prediction of average size distribution and to draw the histogram pattern with obtained data.

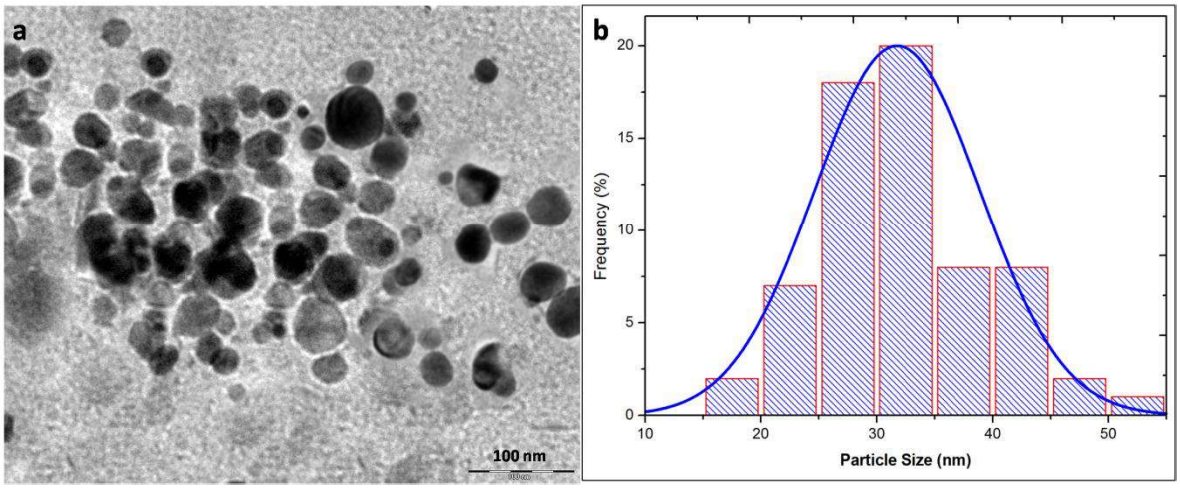

Fig. 5.9 (a) TEM image of AgNPs synthesized at optimum parameters (b) Size distribution histogram and curve for corresponding TEM image

Fig. 5.10 showes the XRD spectra of synthesized AgNPs which represent following diffraction peaks at 2θ =38.46°, 44.17°, 64.72° and 77.67°. The data was well matched with standard pattern of silver (JCPDS file No. 04-0783). These peaks attributed to the (111), (200), (220) and (311) Bragg reflections indicating face centered cubic (fcc) crystal lattice of AgNPs.

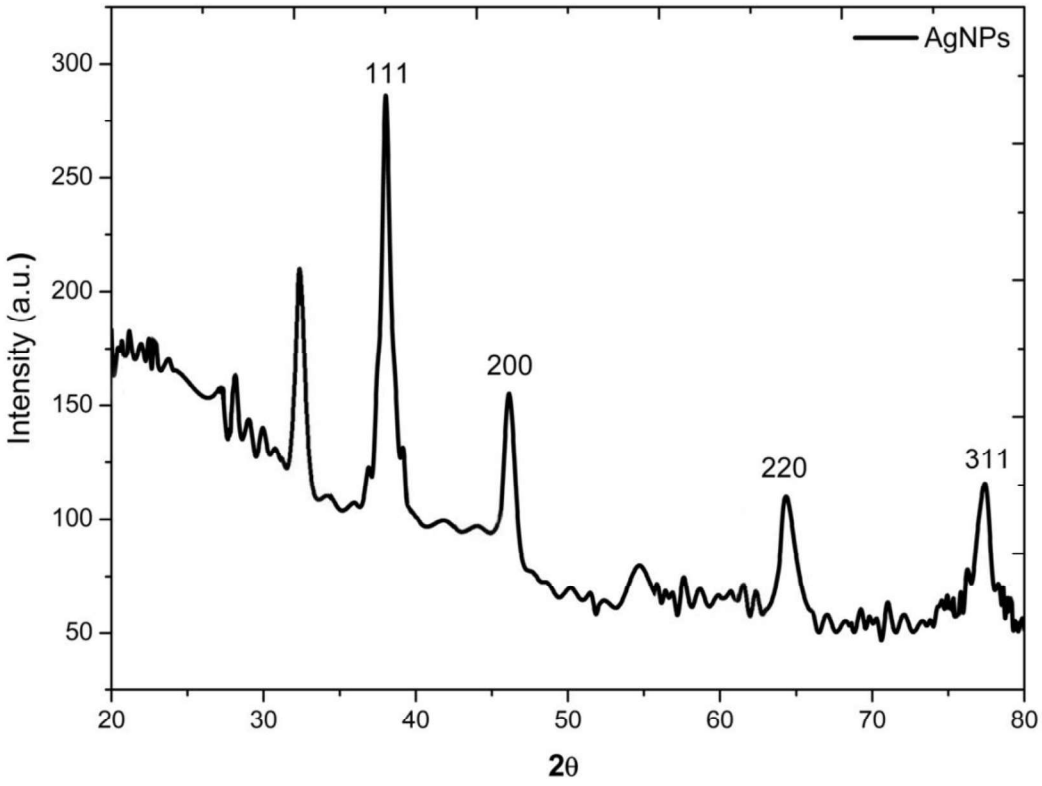

Fig. 5.10 X-ray diffraction pattern of synthesized AgNPs through AET at optimum parameters

FTIR analysis of both AET and AgNPs (*Fig. 5.11*) 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 3200-3400 cm^{-1} which correspond to '–OH' groups of tannin, flavanoides and other phenolic compounds. This broad band (3200-3400 cm^{-1}) also corresponds to '–OH' groups of glucose and '–NH' stretching of the proteins [Bai and Abraham, 2002;

Huang et al., 2009; Nakano et al., 2001]. Peak at 1626.45 cm^{-1} assigned to primary and secondary amines as well as amide linkage of proteins [Nakano et al., 2001; Velmurugan et al., 2013]. The absorbance peak around 1446.63 cm^{-1} attributed to N–H stretching, O–H deformation and $–CH_2$ scissoring [Giri et al., 2011]. Sharp band around 1371.76 & 1035.86 cm^{-1} denotes stretching vibrations carboxylic groups and '–CN' stretching of proteins respectively [Bai and Abraham, 2002; Huang et al., 2009; Nakano et al., 2001; Velmurugan et al., 2013]. IR spectra of AgNPs showed shifting in following peaks and bands as compared with IR spectra of AET: peak at 1626.45 sifted to 1623.23 and 1446.63 to 1404.32 cm^{-1}. An intense narrowing in the broad band (3200-3400 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 [Kuru, 2014].

Stability of AgNPs, synthesized was also monitored upto visual precipitation . The reduction in the SPR band intensity was also measured with proceeding of time. The results are shown in *Fig 5.12*. The AgNPs were found to be stable up to 25 days. After 10 days, turbidity of sample gradually starts to precipitate and finally complete decolonization and precipitation takes place in 30 days.

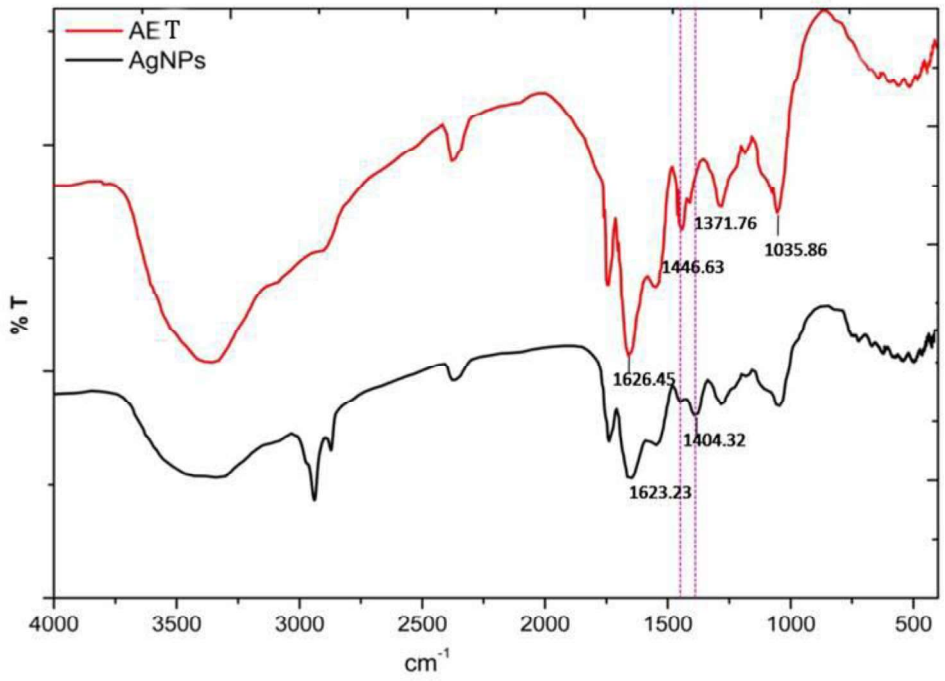

Fig. 5.11 FTIR spectra of the AET and AgNPs

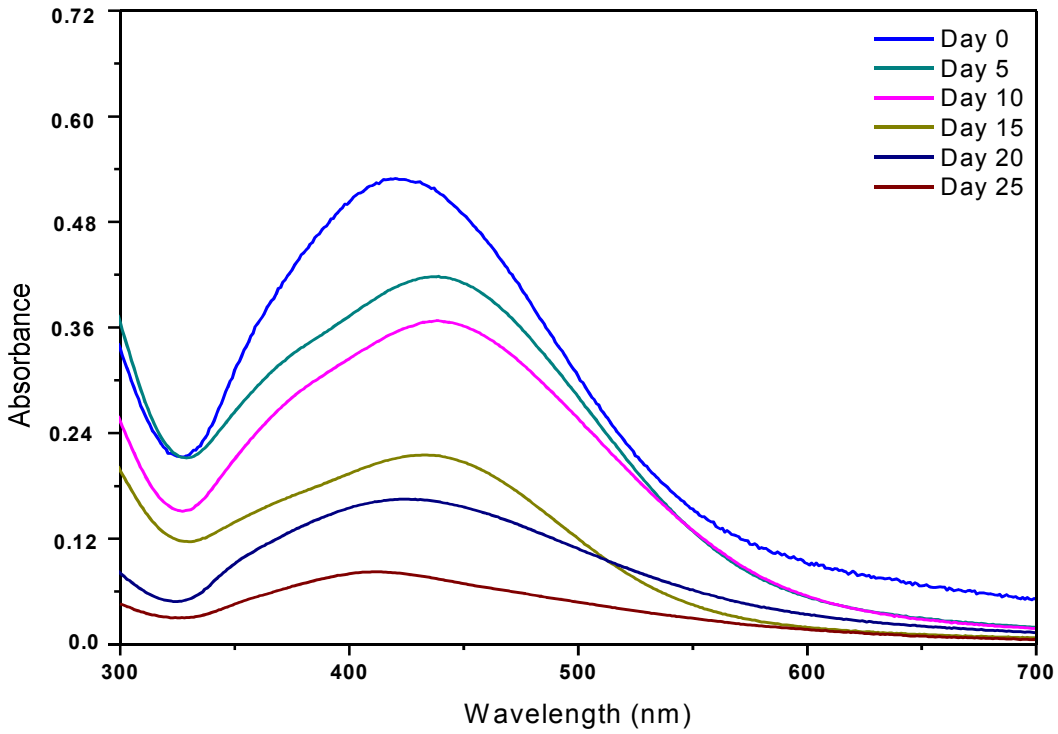

Fig. 5.12 U.V-Visible spectra of AgNPs showing stability of AET synthesized AgNPs up to 25 days of incubation period

5.5.6. 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 5.12 a, b*) which represents zone of inhibitions (ZOIs) around individual discs, inoculated with 20µL suspensions of AgNPs (50 ppm), AET and 5 mM $AgNO_3$ solution and control buffer solution. Measured values of ZOIs are enlisted in *Table 5.1.*

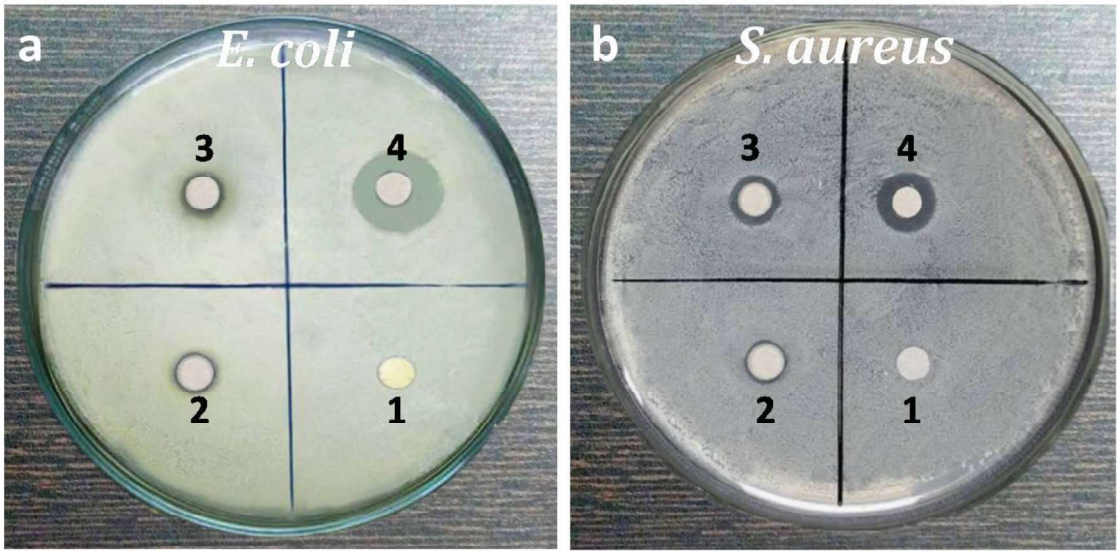

Fig. 5.13 Antimicrobial efficacy of synthesized AgNPs against; (a) *E. coli* (Gram -ve) and (b) *Staphylococcus aureus* (Gram +ve). Disc (1) Buffer solution, (2) AET (3) $AgNO_3$ and disc (4) AgNPs.

Table 5.1 ***Antimicrobial activity of AET, $AgNO_3$ solution and AgNPs against E. coli and S. aureus. Phosphate buffer saline was used as negative control***

Disc	Applied Substance (20 µL)	Zone of inhibition (ZOI) in mm	
		Eischherichia coli	*Staphyloccocus aureus*
1	Buffer Solution (Control)	0	0
2	AET	7	7
3	$AgNO_3$ (5mM)	9	8
4	AgNPs (50 ppm)	18	14

The diameter of ZOIs created by AgNPs on *E. coli* and *Staphylococcus aureus* test plates were 18 mm and 14 mm respectively. Results had proven the significant antibacterial effects of the 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 [Chaloupka et al., 2010]. These findings are in agreement with many previous reports [Kim et al., 2007; Gurunathan et al., 2014; Hungund et al., 2015]. Minor zone of inhibitions were also observed around the discs containing AET and $AgNO_3$. Interaction of free silver ions of $AgNO_3$ with vital enzymes of bacteria provides a mild antibacterial activity to $AgNO_3$ whereas herbal extracts poses some 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 [Jones and Hoek, 2010].

MIC determination for AgNPs was done though amended macro-dilution method [Wendelin, 1997]. MIC was determined 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 getter susceptibility of *E. coli* for AgNPs as compared to *S. aureus*. MIC values further support the results of disc diffusion assay.

5.5.7 Cell viability test

Antibacterial potential of AgNPs against both the test microorganisms i.e. *E. coli* and *S. aureus* was evaluated through cell viability test. The cells of *E. coli* and *S.*

aureus (10^7cells/ml) were incubated in separate MHB media having AgNPs concentration of 16.50 µg/mL and 19.5 µg/mL respectively. Cell viability of both microorganisms was monitored at regular time interval through colony counting method and the results are presented in *Fig. 5.13.*

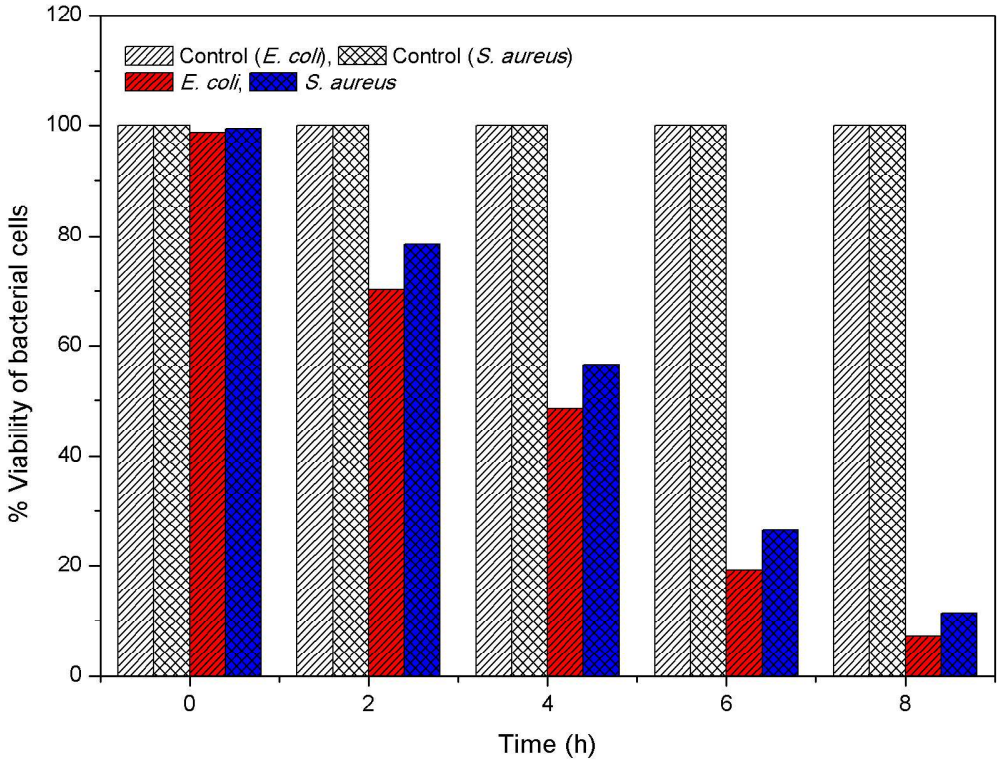

Fig 5.14 Cell-viability test: decrease in number of viable cells of *E. coli* and *S. aureus* (10^7 colony forming units/mL) when treated with AgNPs (conc. equivalent to MIC) at 37 °C for 8 h

The loss of cell viability of *E. coli* and *S. aureus* was counted at bi-hourly intervals. After 8h of incubation with AgNPs, the *E. coli* and *S. aureus* cells showed 92.3 and 88.6% loss loss of cell viability, respectively. There was no loss of cell viability in control sample of *E. coli* and *S. aureus* in both conditions.

5.6 Conclusion of the chapter

AgNPs were successfully synthesized using aqueous extract *Tamarindus indica* (AET) leaves. The developed process was able to initiate the synthesisis

AgNPs within 5 min and complete the synthesis in 40 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.

5.7 Conclusion of the 'Section II'

Both AES and AET were found to be capable of synthesizing the AgNPs in very less time. Detailed studies explored that the AgNPs synthesized through AES have smaller size and greater antimicrobial potential as compared to AgNPs synthesized by AET. Therefore, AgNPs synthesized by AES; were selected for incorporation in to the scaffolds along with *cpr*.