CHAPTER 3

MATERIALS AND EXPERIMENTAL METHODS

3.0 INTRODUCTION

This chapter explains the research procedures employed during the synthesis of AgNPs and the preparation of nanocomposites, as well as experimental strategies for assembling and preparing the various materials used in this study. The experiment's materials and technique were divided into four phases (Figure 3.1) in accordance with its needs.

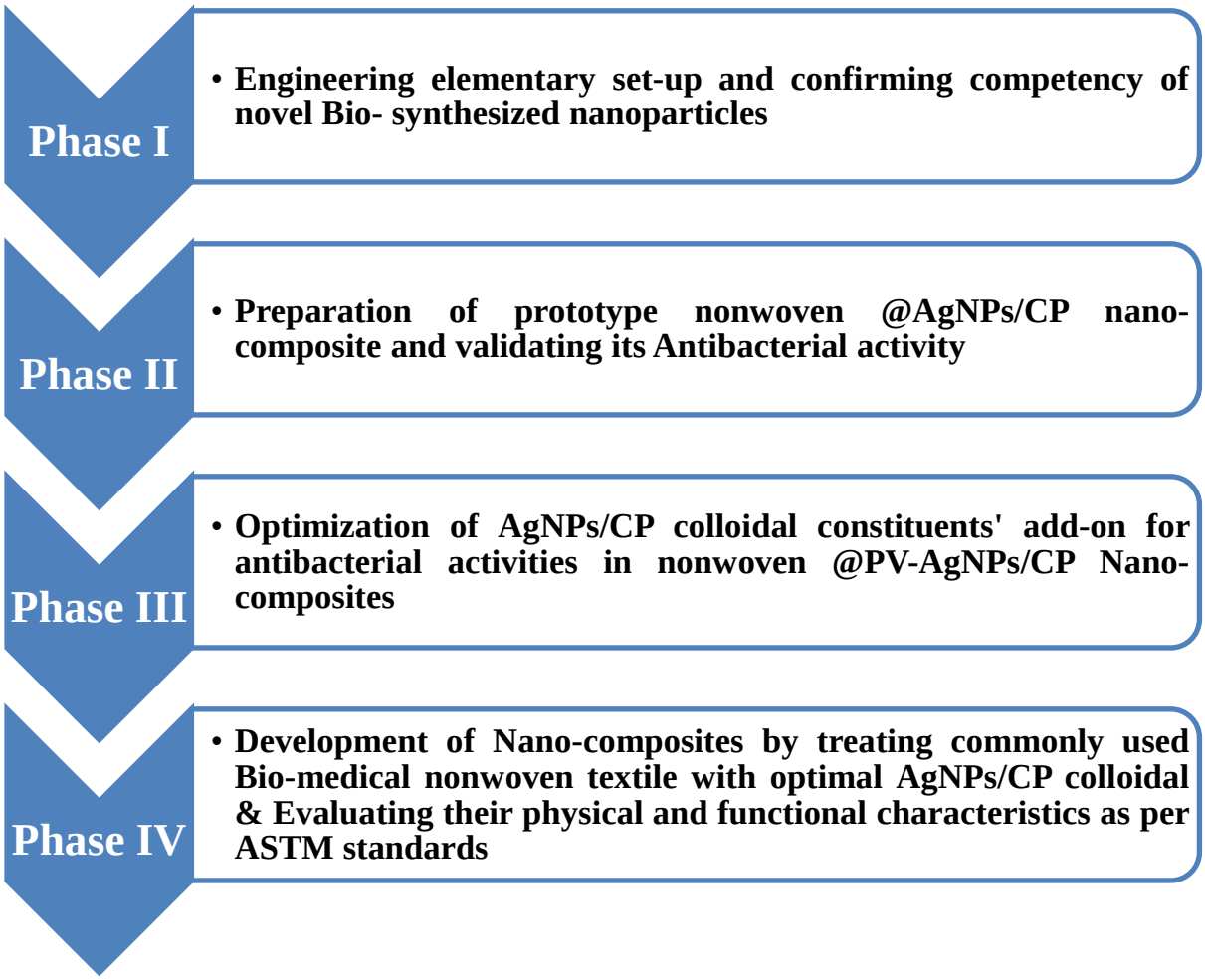


Figure 3.1: Plan of experimental work

3.1 PHASE–I:

ENGINEERING ELEMENTARY SET-UP AND CONFIRMING COMPETENCY OF NOVEL BIO-SYNTHESIZED NANOPARTICLES

Aim: This phase of experiments dealt with engineering novelty technique for AgNPs synthesis with the untouched green medium and defining its competency with the commercially successful chemical gateway.

This set of pilot trials were conducted to synthesized AgNPs from $AgNO_3$ salt by keeping its solution concentration as well as add-on identical.

3.1.1 MATERIALS

***Metal Salt*:** Both the methods necessitate use of basic metal salt. Silver nitrate ($AgNO_3$), 169.87 g/mol was used for the purpose. Used without further purification with ≥ 99.00 % purity prescribed by the supplier.

Capping and Reducing agent: The selected synthesis routes differed in this regard;

- ***Chemical Synthesis (CS)*:** Sodium borohydride ($NaBH_4$), 37.83 g/mol as capping agent, and Tri-sodium citrate ($Na_3C_6H_5O_7$), 257.97 g/mol as reducing agent were used during the study. Their percent purity prescribed by the supplier was; ≥ 97.00, and high respectively and used without further purification.

  All the chemical constituents including metallic salt were procured from reputed supplier; Loba Chemie Pvt. Ltd.
- ***Green Synthesis (GS)*:** Instead of chemicals, CP leave extract was used in green synthesis for capping and reducing agent's combined roles. Based on other green mediums' back track record, CP leave during its newly structured green synthesis process would expect to follow the same pathway [277].

  In the limelight of the observations and literature surveyed for other natural mediums, only two synthesis routes were identified and studied in the elementary phase trials [278,279]. The required CP leave extract was prepared in the controlled environment of the laboratory.

3.1.2 METHODS

3.1.2.1 AgNPs Synthesis methods:

As mentioned in the beginning; the $AgNO_3$ salt, its solution concentration level as well as add-on level were kept identical in both the techniques. However, the supporting constituents' concentrations were differed and their values were purely influenced by the respective back track record. Referencing of optimal composition with various plant extracts; neem [280], aloe vera [281,282], tulsi [3], etc. derived in AgNPs synthesis using the same base salt; $AgNO_3$ was made in case of the novel green route [283].

3.1.2.1.1 Chemical synthesis (CS) technique:

The chemical AgNPs synthesis process has acquired commercial significance till date on the account of its well proven effectiveness, controlled NPs size and high production rate mainly [284]. Three synthesis routes are normally used in this technique;

i) Forward synthesis method (Top-down approach),
ii) Reverse synthesis method (Bottom-up approach), and
iii) Reduction method (with Tri-sodium citrate).

All the three ways are similar in terms of processing steps and concentration levels used for the constituents but they differ only in terms of constituents' add-on values. Accordingly samples CS-1, CS-2 and CS-3 were prepared (Table 3.1).

Table 3.1: Constituents' add-on levels during AgNPs synthesis

Colloidal Solution	Silver nitrate ($AgNO_3$)	Sodium Borohydride ($NaBH_4$)	Tri-sodium citrate ($Na_3C_6H_5O_7$)	Calotropis leave extract (CP)
		1. Chemical synthesis (CS)		
CS-1 (Forward Method)	100 ml	10 ml	5 ml	-
CS-2 (Reverse Method)	10 ml	100 ml	5 ml	-
CS-3 (Reduction Method)	100 ml	10 ml	20 – 30 ml	-
		2. Green synthesis (GS)		
GS-1 (Forward Method)	100 ml	-	-	10 ml
GS-2 (Reverse Method)	10 ml	-	-	100 ml

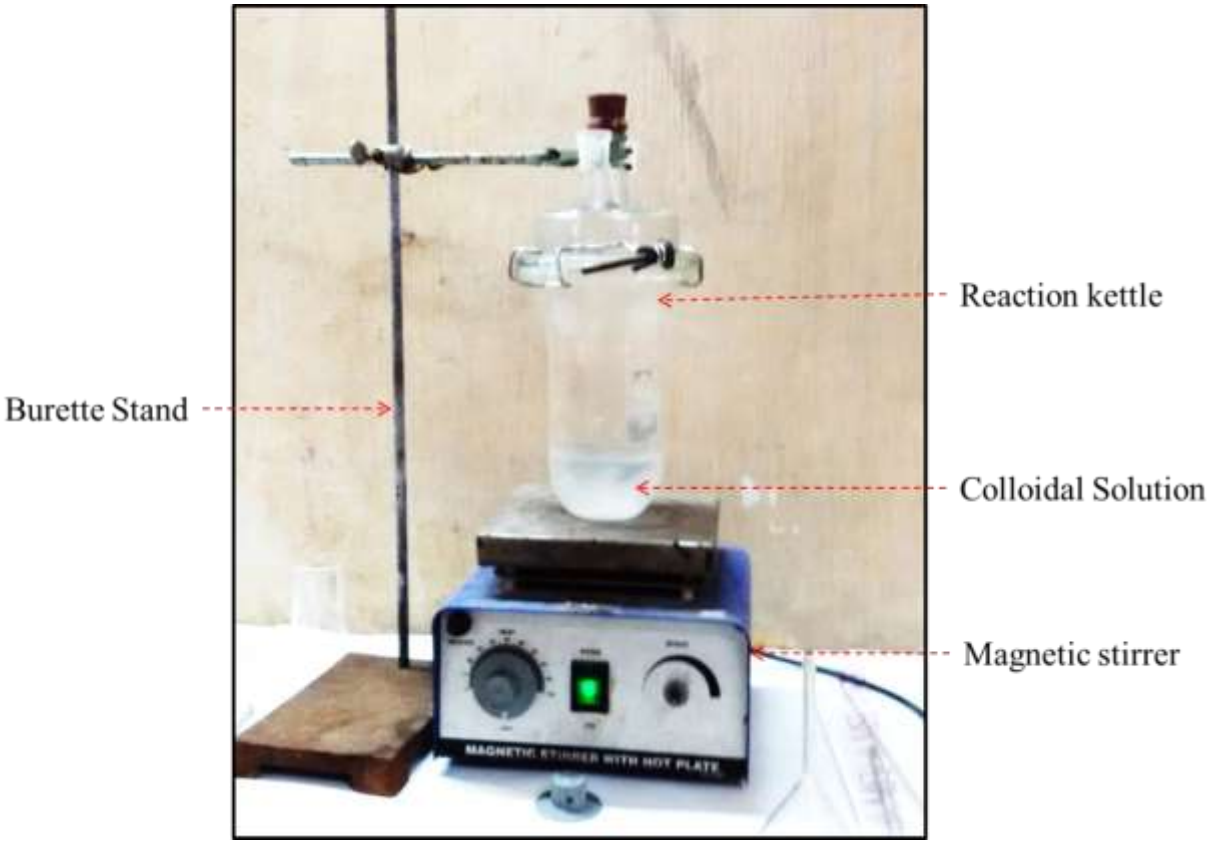


Figure 3.2: Experimental lab set-up for Chemical synthesis

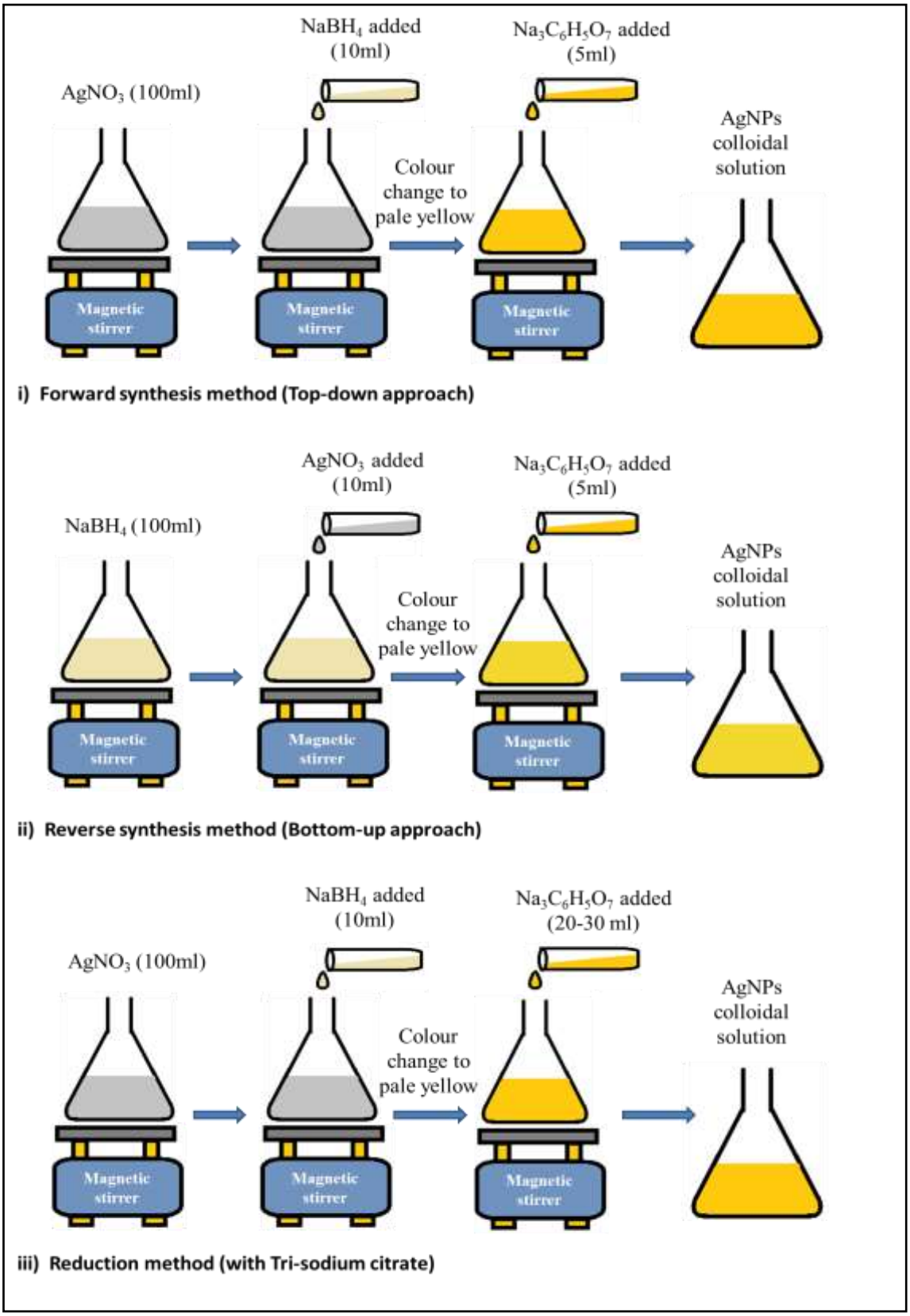


Figure 3.3: Schematic illustration of Chemical AgNPs synthesis methods

Since, identical procedures were used in the preparation of $AgNO_3$, $NaBH_4$ and $Na_3C_6H_5O_7$ solutions as well as in AgNPs synthesis; they are described in general to avoid undue duplication. Even reverse synthesis method differs from the rest of two methods at first step, only, so description of that step is given separately.

Preparation of Solutions:

a. ***$AgNO_3$ solution [10mM (1%) concentration]:*** Precise weight of 0.169 g base salt; $AgNO_3$ powder was added into 100 ml solvent [double distilled water (DDW)] to form a $AgNO_3$ solution of 10 mM (or 1%) concentration. This solution was then kept in the reaction chamber and vigorously stirred for 30 minutes at room temperature by using a magnetic stirrer to avoid lump formation.
b. ***$NaBH_4$ solution [30mM (3%) concentration]:*** It was prepared by adding 0.1 g powder to 100ml DDW along with continuous stirring.
c. ***$Na_3C_6H_5O_7$ solution [10mM (1%) concentration]:*** It was prepared by adding 0.26 g powder to 100ml DDW along with continuous stirring.

Chemical synthesis of AgNPs:

The lab set-up used in the chemical synthesis method is shown in Figure 3.2 and stepwise procedure illustrated schematically in Figure 3.3. Each variant's prescribed quantity was taken as per the selected method (Table 3.1).

i) Forward and Reduction method: The base salt solution; 10mM $AgNO_3$ was taken into the reaction kettle and reduction of silver particles was carried out by gradually adding aqueous chilled solution of 30mM $NaBH_4$ respectively via pipette.

ii) Reverse method: 30mM $NaBH_4$ was taken into the reaction kettle and reduction of silver particles was carried out by gradually adding 10mM $AgNO_3$.

The solution colour when started turning to pale (light) yellowish (on the formation of NPs), 10 mM concentrated capping agent; $Na_3C_6H_5O_7$ solution (to impart stability) were added (respectively as per the method of preparation) drop by drop with constant stirring.

Prepared AgNPs colloidal solutions were kept for colour change observation as per the preliminary AgNPs confirmation method, widely used by the researchers [285].

3.1.2.1.2 Green synthesis (GS) technique:

The green AgNPs synthesis was done by two synthesis routes, viz;

i) Forward synthesis method (Top-down approach), and

ii) Reverse synthesis method (Bottom-up approach).

Similar to chemical methods both the green methods also differ in terms of proportionate constituent add-on only. Accordingly, samples GS-1 and GS-2 were prepared (Table 3.1). Preparation of $AgNO_3$ (10mM concentration) solution was done in the same manner to the chemical synthesis. Thereby, method followed in the preparation of CP leave extract is only described in this section.

Preparations of Calotropis (CP) leave extract

This laboratory technique was influenced by the procedure followed in the preparation of natural matter functional extract [2,3]. It is graphically illustrated in Figure 3.4. The formula involved two steps;

i. Preparation of CP leave powder, and

ii. Preparations of CP leave extract.

i) Preparation of the powder

Fresh and healthy CP leave (around 1 kg) were collected from the randomly selected plants, grown abundantly in the nearby river bank area. These leave were washed twice with tap water to remove debris, dirt, and other contaminations, followed by a final wash with DDW. This was done to get rid of residual contaminations arisen from tape water. Subsequently, the leave were oven dried at 70^oC ($\pm$ 5^oC) for 24 hours. This slow rate drying of leave for long time interval was preferred to avoid loss of inbuilt substances or their thermal damage [286]. Such dried leave were then crushed, grinded into a fine powder and stored into vacuum sealed polyethylene bags. This powder was used in the preparation of required concentration of extract.

ii) Preparation of the extract

The CP leave (100 g) powder was soaked into (1000 ml) DDW filled in sealed glass vessel and heated at 70^oC ($\pm$ 5^oC) for two hours. After that, the mixture was cooled, and filtered with Whatman filter paper no.-1 to remove clustered particles. The prepared 10% concentration CP leave extract was then used in the green synthesis of AgNPs.

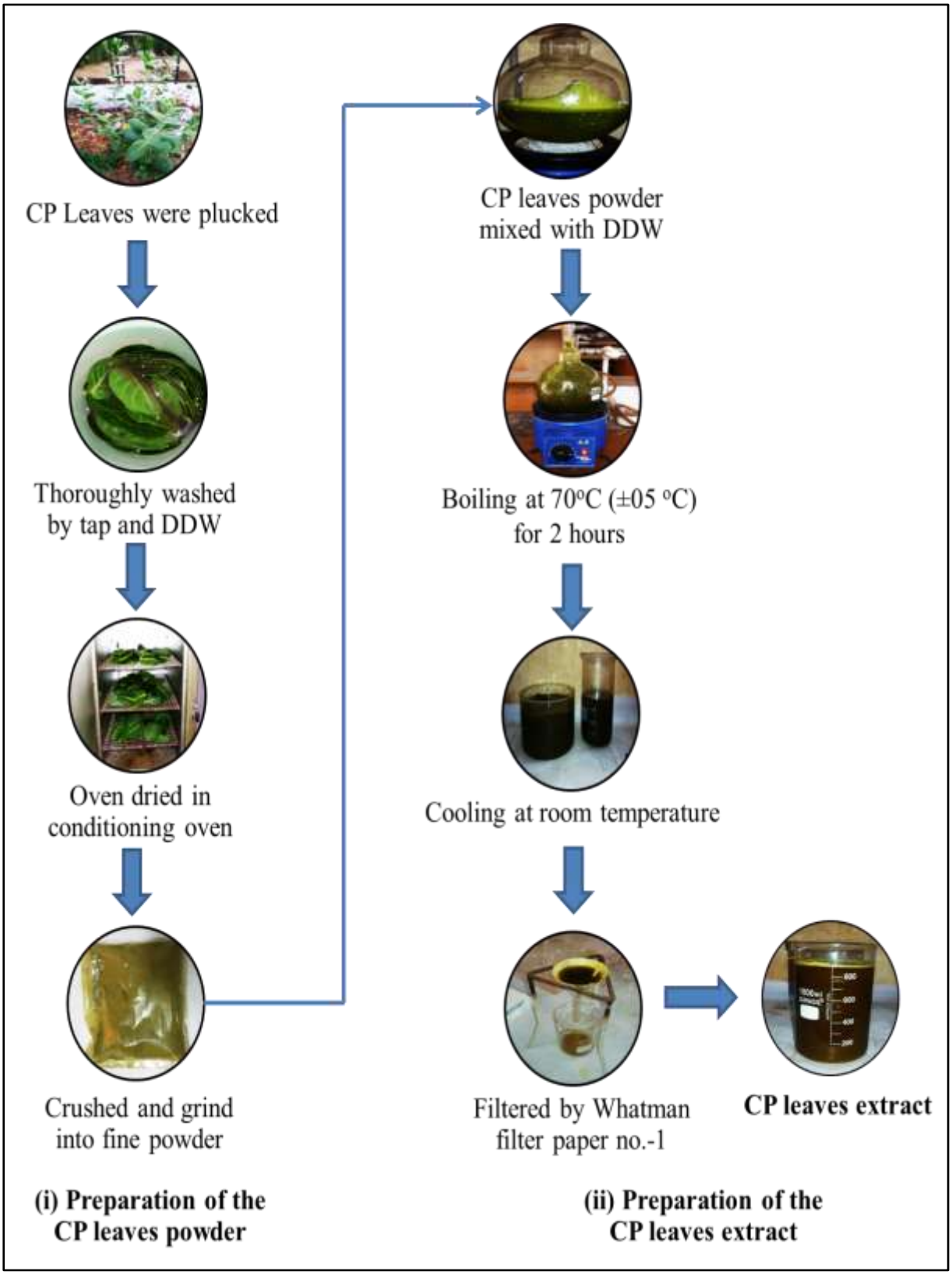


Figure 3.4: Preparation of the CP leave powder and extract

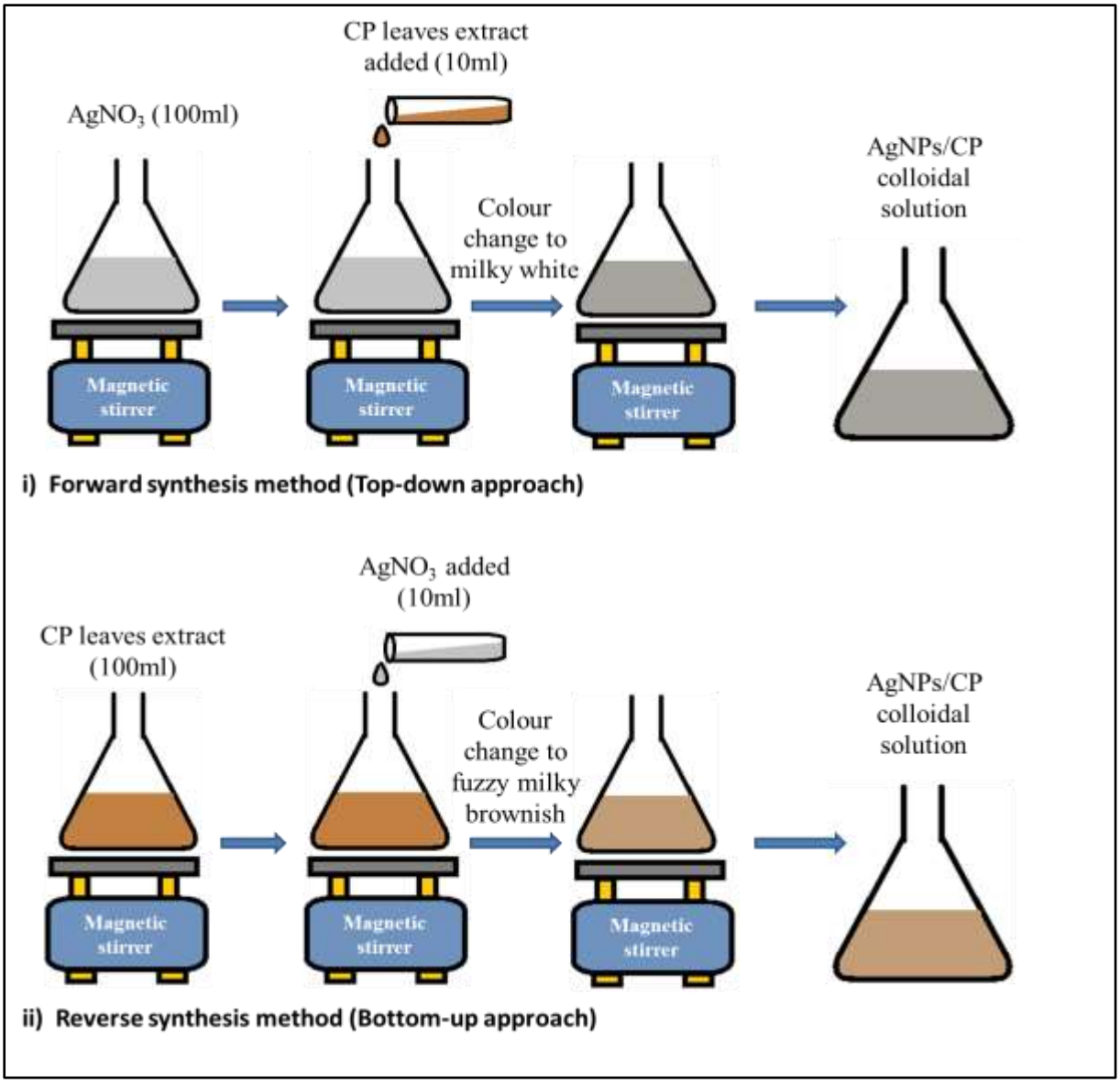


Figure 3.5: Green synthesis methods of AgNPs

Green synthesis of AgNPs:

Similar to chemical synthesis processing steps except the first one for both the green methods were same, only changing in terms of add-on values of constituents (Table 3.1). So, in order to avoid duplication, generalized descriptions for the steps followed in both the methods are incorporated here. The lab set-up used in the chemical synthesis (Figure 3.2) was utilized for green synthesis also. The schematic flow of the green synthesis methods are illustrated in Figure 3.5.

i) Forward method: The base salt solution; 10mM $AgNO_3$ was taken into the reaction kettle. Reduction and capping of silver particles was carried out with 10% concentrated CP leave extract added drop by drop via pipette. Continuous stirring was done while adding the extract to avoid any type of clump formation.

ii) Reverse method: 10% concentrated CP leave extract was taken into the reaction kettle. This has caused reduction and capping of silver particles of 10mM $AgNO_3$ solution added drop by drop along with continuous stirring.

The prepared AgNPs colloidal solutions were kept for colour change observation as per the preliminary AgNPs confirmation method, widely used by the researchers [285].

3.1.3 TEST METHODS

The outputted colloidal produced via both the routes were verified for the formation of nanoparticles and their stability period. The established visual colour change technique widely adopted for elementary confirmation of AgNPs formation was used [233].

However, SEM analysis was additionally done for both the green synthesized AgNPs colloidal. This was required due to presence of dominating natural light brown colour of the CP leave extract in the colloidal, which has caused interference during this visual judicial colour measurement and added difficulty level in precise conclusion.

Thence after, out of the two green routes experimented; the sorted best method was used for further study.

3.1.3.1 Visual colour change observation technique (VCCO)

The colloidal has been observed with defined time creep; after 0 hour, 24 hours, and 48 hours for confirmation and shelf life of AgNPs or say stability. The criterion for the analysis as depicted is the colour observed for the colloidal under study. If it executes light yellowish tint indicates presence of nanoparticles. But if the colloidal tint from initial yellowish turns into dark brownish/blackish tint with respect to time giving the indication about clustering of nanoparticles and formation of macro molecules, i.e. poor shelf life or unstable AgNPs formation [233].

3.1.3.2 Surface morphology analysis by Scanning Electron Microscopy (SEM)

The Scanning Electron Microscope (SEM), Model JSM-5610 LV, Version 1.0, Jeol Japan (Figure 3.6) was used to characterize the AgNPs, by using Oxford-Inca software.

Sample was prepared by putting a drop of the green synthesized AgNPs colloidal solution on carbon coated aluminium sheet followed by drying at room temperature for 15 minutes. It was illuminated under the scanning electron microscope and scanned images produced at 1000X and 1600X magnifications with 100Å (angstrom) resolution were recorded on an interfaced computer.

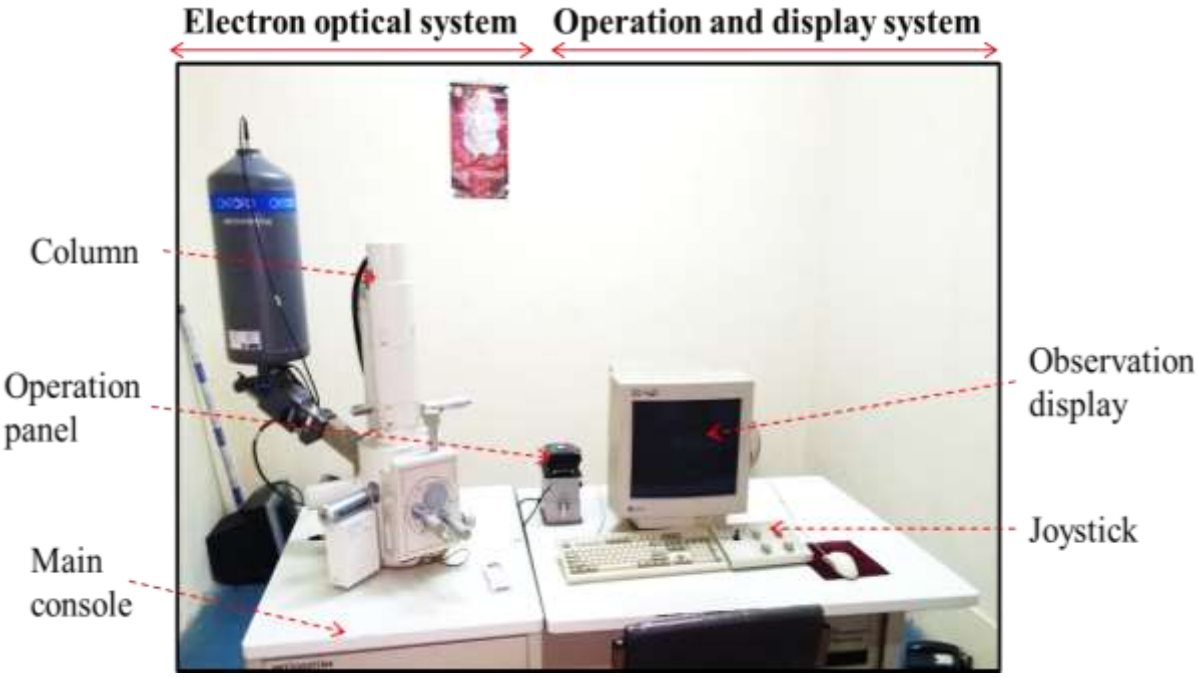


Figure 3.6:

Scanning Electron Microscope (Model JSM-5610 LV, Version 1.0, Jeol Japan)

3.2 PHASE–II:

PREPARATION OF PROTOTYPE NONWOVEN @AgNPs/CP NANO-COMPOSITE AND VALIDATING ITS ANTIBACTERIAL ACTIVITY

Aim: This phase of study was designed to prepare prototype nano-composite with innovative green-way synthesized AgNPs colloidal in Phase-I and evaluating its antibacterial efficacy.

The nanoparticles formation and their stability using CP leave extract as reducing and capping agent were analysed in the previous phase. During the pilot trials, the reverse method i.e. GS-2: Bottom-up approach (Table 3.1) has shown promising nanoparticle formation with good stability. Therefore, the nonwoven nano-composite was prepared using this AgNPs colloidal (GS-2) and evaluated for its antibacterial efficacy.

3.2.1 MATERIALS

Preparation of the nano-composite has included use of two elements, viz;

i) **Base textile material (Fabric):** Nonwoven fabric composed of 70:30 Polyester-Viscose (PV) blend and having 40 gram per square meter (GSM) and 0.4 mm thickness was used to entrap antibacterial nanoparticles. Comfort characteristics, recyclable, moderate strength, low bending modulus, and above all lowest cost amongst all types of textile fabrics (woven, knitted and nonwoven) and used popularly for targeted bio-medical purposes (surgical aprons, medical drapes, bandages, gloves, gowns etc.) are the major criterion considered in this selection [1].

ii) **Antibacterial medium:** AgNPs colloidal synthesized in the first phase of experimentation with green channel via Bottom-up approach (GS-2) was used to create required antibacterial reagent in the selected fabric structure.

Antibacterial characterization of the engineered PV-nonwoven nano-composite @PV-AgNPs/CP has required formation of nutrient agar medium.

iii) **Nutrient agar medium**: Agar-agar powder for bacteriology grade and nutrient broth for general cultivation of less fastidious microorganisms were procured from the reputed Loba Chemie Pvt. Ltd. and HiMedia Laboratories respectively. They were used in the laboratory scale preparation of the required nutrient agar culture/medium. Their specifications were chosen as defined in standard test procedure [265]. The pH value for agar-agar powder was 5 – 7 and for nutrient broth 7.4 ± 0.2. Both the

materials were remains in solid state at around 20^{o}C. Nutrient broth contains peptone (5 gpl), sodium chloride (5 gpl), meat extract B# (1.5 gpl), and yeast extract (1.5 gpl).

3.2.2 METHODS

Defining the extent of antibacterial efficacy inhabited into the base PV-nonwoven fabric on novel green synthesized AgNPs treatment was the objective of this phase. Thereby two types of PV-nonwoven fabrics were considered during evaluation, viz;

1) Reference Sample 00: untreated PV-nonwoven, and

2) Prototype Sample 01: PV-nonwoven nano-composite @PV-AgNPs/CP.

Preparation of Prototype PV-AgNPs/CP nano-composite (Sample 01)

The nano-composite textile material (PV-AgNPs/CP) was made in the laboratory by treating the base fabric with the best one sorted out of the two new green-way synthesized AgNPs colloidal in Phase-I, viz; GS-2 (Table 3.1). The cold dipping technique was used for colloidal application (Figure 3.7). The known weight (~2 g) of rectangular (25 × 20 cm) base fabric sample was dipped into the beaker filled with AgNPs/CP colloidal solution by maintaining constant 1:25 bath ratio (1 g of the sample dipped into 25 ml colloidal solution) for 02 hours at room temperature [287]. After that, the sample was dried by hanging freely at room temperature and preserved in a vacuum sealed plastic bag till further analysis.

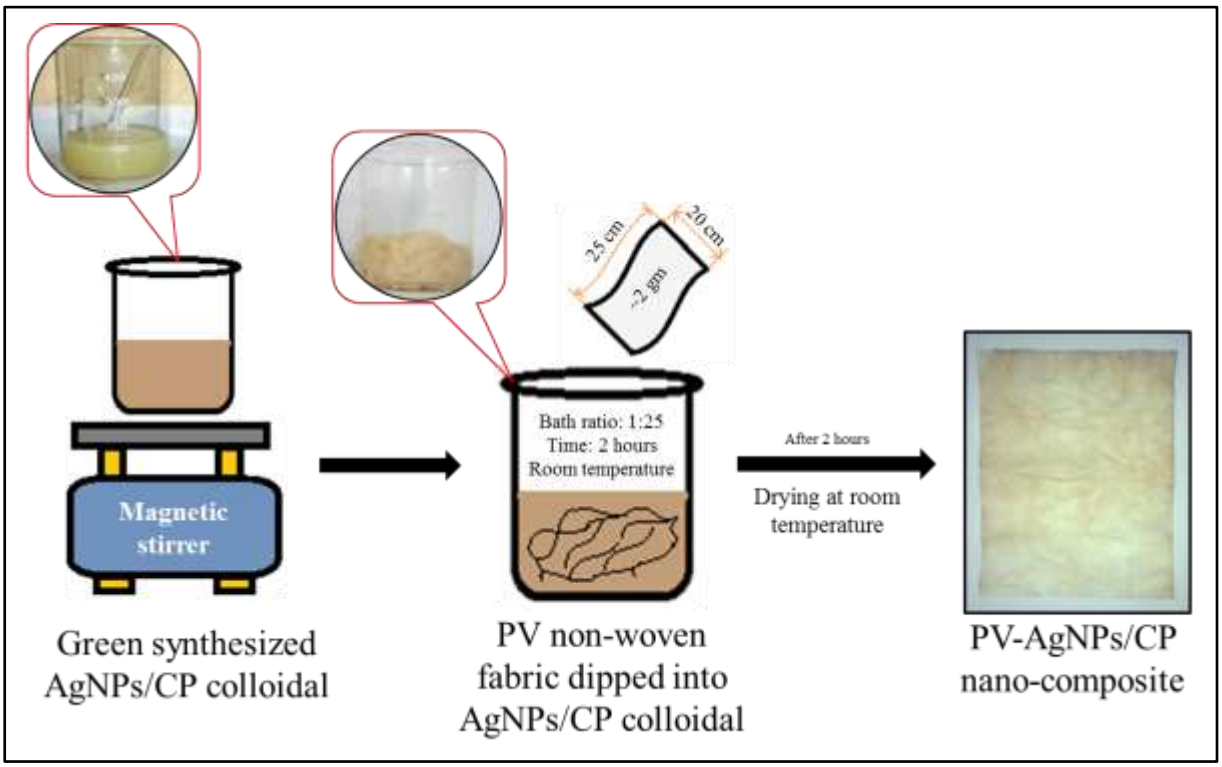


Figure 3.7: Schematic illustration of preparation of nano-composite textile material.

3.2.3 TEST METHODS

The prepared prototype PV-AgNPs/CP nano-composite (sample 01) was analyzed for its antibacterial activities with reference to untreated sample 00. Consistency and proficiency of the nano form antibacterial reagent (AgNPs) is influenced by the characteristics and extent of uniform distribution of NPs into the composite structure [287]. Thereby these measures were also taken for the prototype composite in this phase. Apart from this influence of novel AgNPs/CP treatment on the bio-medically important base fabric characteristics, viz; physical, low-stress and comfort-related characteristics; were assessed. The samples were conditioned at the standard atmospheric conditions for tropical region; (Temp: $27^{o}C \pm 2^{o}C$ and RH% = 65 ± 2) for 24 hours before testing. The standard evaluation techniques used are listed below:

a) AATCC-147 for Antibacterial assessment
b) Structural and morphological assessment for AgNPs
   i) Environmental Scanning Electron Microscopy (ESEM)
   ii) Energy Dispersive X-Ray Spectroscopy (EDAX)
   iii) Size distribution of nanoparticles by using 'ImageJ' Software
   iv) Fourier Transforms Infrared Spectroscopy (FTIR) analysis
c) Physical and comfort-related properties
   i) Physical assessment: GSM (gram per square meter), and Thickness
   ii) Low-stress properties: Bending modulus, and Crease recovery angle
   iii) Comfort-related properties: Air permeability, and Overall moisture management capability (OMMC)
d) UV Transmission properties

3.2.3.1 AATCC-147 test methods for Antibacterial assessment

The antibacterial assessment was done by parallel streak test method [265]. The antibacterial activity was checked against two pathogens, viz; Staphylococcus aureus (NCIM 2654) - gram positive and Escherichia coli (NCIM 2832) - gram negative [265]. The method is useful in obtaining a rough estimate of activity in that the growth of the inoculum organism decreases from one end of each streak to the other and from one streak to the next streak, resulting in increased degrees of sensitivity. The zone size of inhibition (ZOI) and the extent of narrowing of the streaks caused by the presence of the antibacterial agent permits an

estimate of the residual antibacterial activity after multiple uses [265]. The test procedure included following steps;

Preparation of bacterial culture medium:

i. Preparation of nutrient agar media
ii. Preparation of Petri dishes for testing
iii. Preparation and maintenance of bacterial culture medium
iv. Loading of bacterial test culture into Petri dishes
v. Testing and Antibacterial assessment

i. Preparation of the nutrient agar media:

Agar media/broth is a nutrient media for bacterial growth. The nutrient agar media was prepared by dissolving 3.0 g (3%) of agar-agar powder and 1.3 g of nutrient broth into 100ml of DDW. The mixture was heated along with constant stirring for complete dissolution of the suspended particles. The mixture was then autoclaved at 103 kPa (15 psi) pressure and 121^{o}C temperature for 15 minutes, followed by cooling at room temperature.

Steps followed in the preparation of the nutrient agar media are graphically abstracted in Figure 3.8.

ii. Preparation of Petri dishes for testing:

Dispense the cooled and sterilized nutrient agar by pouring 20 ± 2 ml quantity into each standard flat bottom Petri dish and then leaving all such filled dishes on the sterile surface for 5 – 10 minutes until the gel form agar media got solidified completely. The petri dishes with solidified agar media were kept for UV stabilization for around 2 minutes. Each petri dish was labelled as per the kind of bacterial culture to be loaded for testing in the next step (Table 3.2).

iii. Preparation of bacterial culture mediums:

Two bacterial cultures were selected for the antibacterial testing of the PV-AgNPs/CP nano-composite;

1. Gram-positive - Staphylococcus Aureus (NCIM 2654)

2. Gram-Negative - Escherichia coli (NCIM 2832)

Both the bacterial inoculums were prepared and maintained in the same manner as described stepwise below;

Bacterial inoculum Preparation: The sterile nutrient broth solution was prepared by dissolving 1.3 gm of nutrient broth powder into 100ml of DDW. Such 9.0 ± 0.1 ml of sterile nutrient broth solution was taken per bacterial culture tube. Ten bacterial culture tubes were prepared out of the solution followed by sterilization (autoclave) at 103 kPa (15 psi) and 121^{o}C temperature for 15 minutes. These tubes were then preserved in the refrigerator till further use.

1.0 ± 0.1 ml of 24 hours or more maintained one of such broth bacterial culture was taken into a test tube and its bacterial content was diluted by the addition of freshly prepared 9.0 ± 0.1 ml of sterile nutrient broth (NB) solution and mixed well using appropriate agitation for 24 hours before use (Figure 3.9). These steps is required for the daily maintenance of the bacterial culture concentration as depicted by the standard and referred as maintenance of bacterial culture usually [265].

iv. Loading of bacterial test culture into petri dishes:

One loop full of the diluted inoculum (1.0 ± 0.1 ml of 24 hours maintained bacterial culture into 9.0 ± 0.1 ml of sterile DDW with appropriate agitation) was loaded on the UV stabilised Petri dishes by using a 4 mm inoculating wire loop (Figure 3.10). The bacterial inoculums were transferred to the surface of the respective sterile agar plates to make several streaks without refilling the loop. Precaution was taken not to break/damage the agar surface while making the streaks.

Table 3.2: Antibacterial test samples details

Petri dish	Bacterial culture	Sample	
(B-01)	(Staphylococcus Aureus)	00 01	
(B-02)	(Escherichia coli)	00 01	

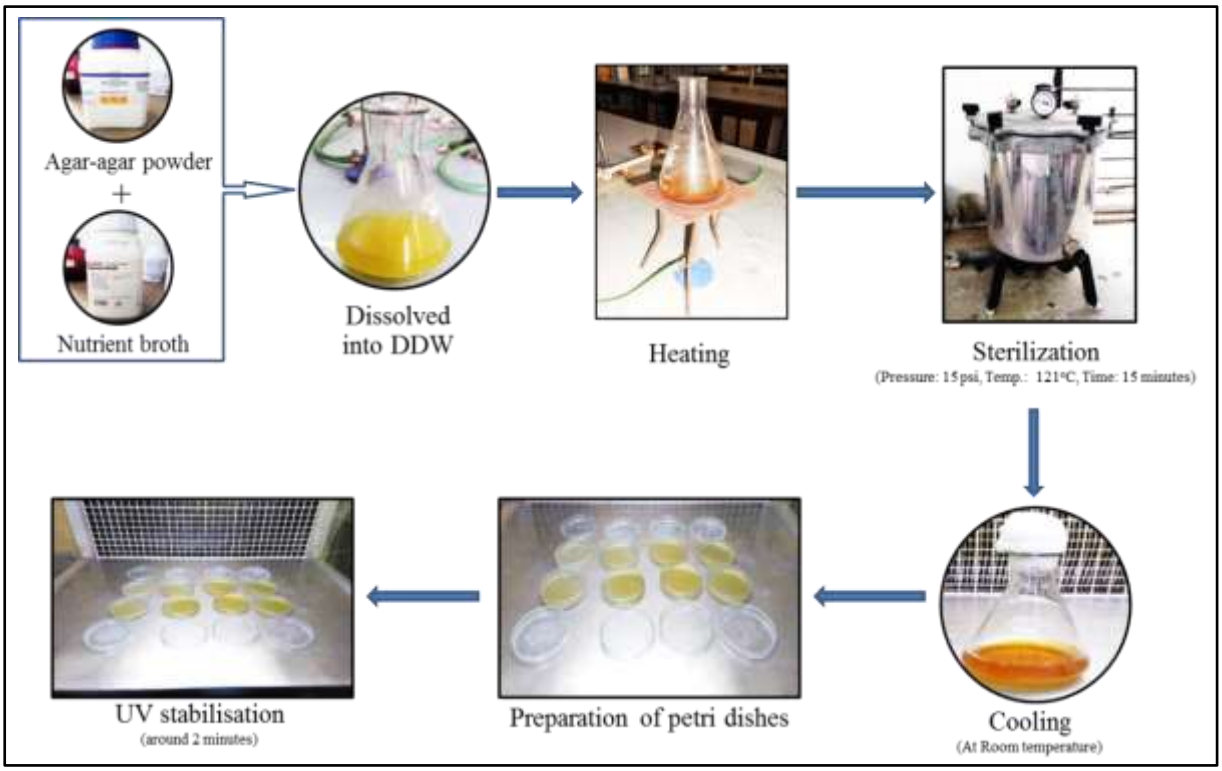


Figure 3.8: Preparation of the nutrient agar media

Figure 3.9: Preparation and Maintenance of bacterial culture

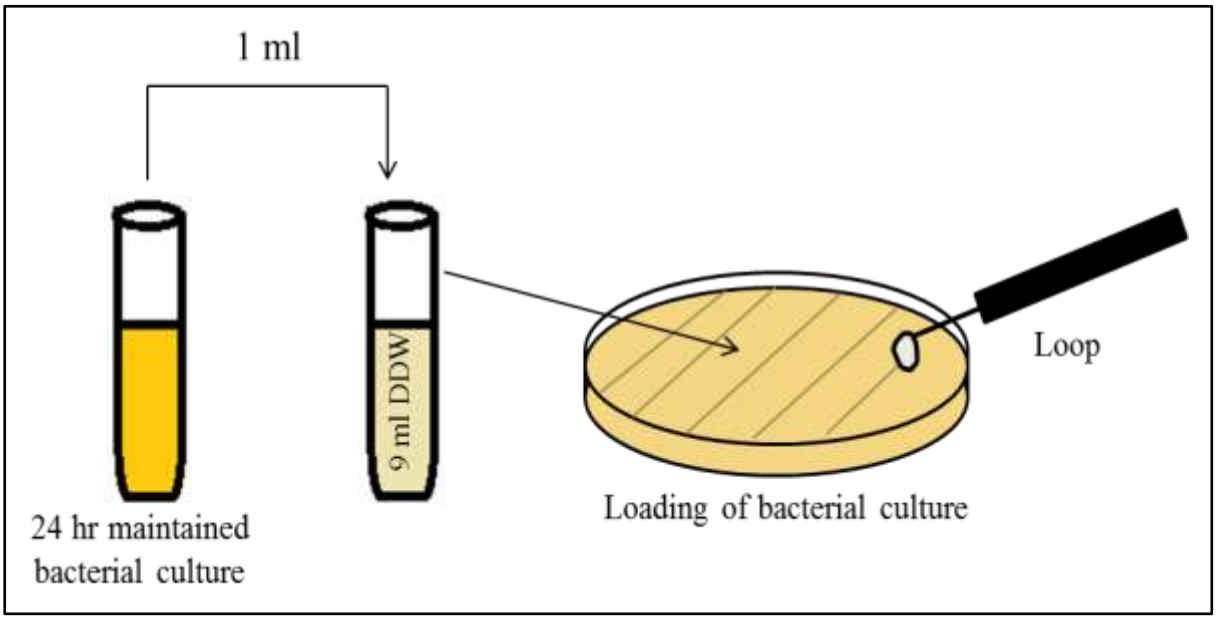


Figure 3.10: Loading of bacterial culture

v. Antibacterial assessment

The antibacterial efficacy on treatment of sample 01 (PV-AgNPs/CP) was judged with respect to control/reference; untreated base (PV non-woven) sample 00. Both the reference and treated samples were cut into two equal sizes rectangular 50×10 mm strips for the selected two bacterial cultures. Here selection of the sample length was done in such a way that it can cover up all the streaks of bacterial culture loaded on the Petri dish. The sample strips were then placed onto nutrient agar media streaked with respective inoculum of test bacteria (Table 3.2). The test specimen was gently pressed in transverse direction across the inoculum streaks to ensure intimate contact with the agar surface. This was done because; if the specimen gets curled, it can avert intimate contact with the inoculated surface, and not allow meeting the compulsory condition for the precision of the test. Sterile glass slide was placed on the ends of the specimen to hold it in place.

The plates were then incubated at 37^{o}C for 24 hours to check the bacterial inhibition. After 24 hours, a clear area without bacteria known as Zone of Inhibition (ZOI) was measured with the scale for each streak. It was occurred as a result of diffusion of an antimicrobial agent from the specimen.

Similarly ZOI (Zi) was measured for all the streaks per test specimen under the study. The average width of ZOI along a streak on either side of the test specimen was then calculated using the following equations:

$$\textbf{Average width of zone of inhibition (ZOI) in mm} = \frac{\sum_{i=1}^{n} Z_i}{n} \quad \ldots\textbf{(3.1)}$$

Coefficient of variation (CV %) and standard deviation of ZOI between streaks was calculated as follows:

$$\textbf{Coefficient of variation (cv)}\% = \frac{\text{standard deviation } (\sigma_{n-1})}{\text{Average } (\bar{X})} \times 100 \quad \ldots\textbf{(3.2)}$$

Where,

$$\textbf{Standard deviation } (\sigma_{n-1}) = \sqrt{\sum \frac{(X-\bar{X})^2}{(n-1)}} \quad \ldots\textbf{(3.3)}$$

x = observed values per streak; $\bar{X}$ =mean ZOI values; n = no. of observations (streak)

The results of untreated sample 00 and the PV-AgNPs/CP nano-composite (sample 01) were compared to define the efficacy of antibacterial treatment and their dispersion.

3.2.3.2 Structural assessment

3.2.3.2.1 Morphological assessment by Environmental Scanning Electron Microscopy (ESEM)

The morphological assessment of untreated PV-nonwoven textile material (sample 00) and PV-AgNPs/CP nano-composite (sample 01) was done by using ESEM (Environmental Scanning Electron Microscope, Model: ESEM EDAX XL-30, Philips, Netherlands) (Figure 3.11). Square samples of approximately 1 cm^2 were cut and placed on carbon coated aluminium sheet, then illuminated under the scanning electron microscope. Scanned ESEM images for both the samples taken at 2nm resolution with 5.0 kV accelerating voltage and magnification levels; 1000 X, 2000 X, 4000 X and 8000 X, were recorded on the interfaced computer. They were used for the deposition confirmation and measurement of shape and size of AgNPs present in the PV-AgNPs/CP nano-composite (sample 01) structure.

3.2.3.2.2 Elemental assessment by Energy Dispersive X-Ray Spectroscopy (EDAX)

The elemental assessment and mapping of the untreated PV-nonwoven textile material (sample 00) and PV-AgNPs/CP nano-composite (sample 01) were performed to report the presence of elements. The samples prepared for ESEM measurement were simultaneously tested for EDAX on the same Environmental Scanning Electron Microscope (Model: ESEM EDAX XL-30, Philips, Netherlands) (Figure 3.11). The Energy dispersive spectrums for the selected area of the samples (00 and 01) were recorded in graphical (elemental curve) and numerical form. The presence of elements in terms of the location and atomic weight of metal particles produces an elemental curve of different metals present in the test sample as pure and oxide state qualitatively. The results of the EDAX were used for comparison of the presents of elements in both the samples.

3.2.3.2.3 Size distribution of nanoparticles

The average nanoparticle size of the AgNPs was analyzed by processing the 8000X ESEM micrograph of the PV-AgNPs/CP nano-composite using 'ImageJ' Software version: 1.53a. This software determines area of the nanoparticles in terms of pixel and also converts it to nanometers by applying scale on the picture. Microsoft Excel software was then used to calculate NPs diameter (Equation 3.4) as per their measured area by the 'ImageJ' Software by considering that cross sectional shape of the nanoparticles is circular [288]. Figure 3.12 represents schematic flow of the ESEM image processing by 'ImageJ' software.

$$\boldsymbol{A} = \frac{\pi}{4} \times \mathrm{D}^2 \qquad \ldots\textbf{(3.4)}$$

Where,

A is the area of the circle in nm^2, D is the diameter of the nanoparticles in nm.

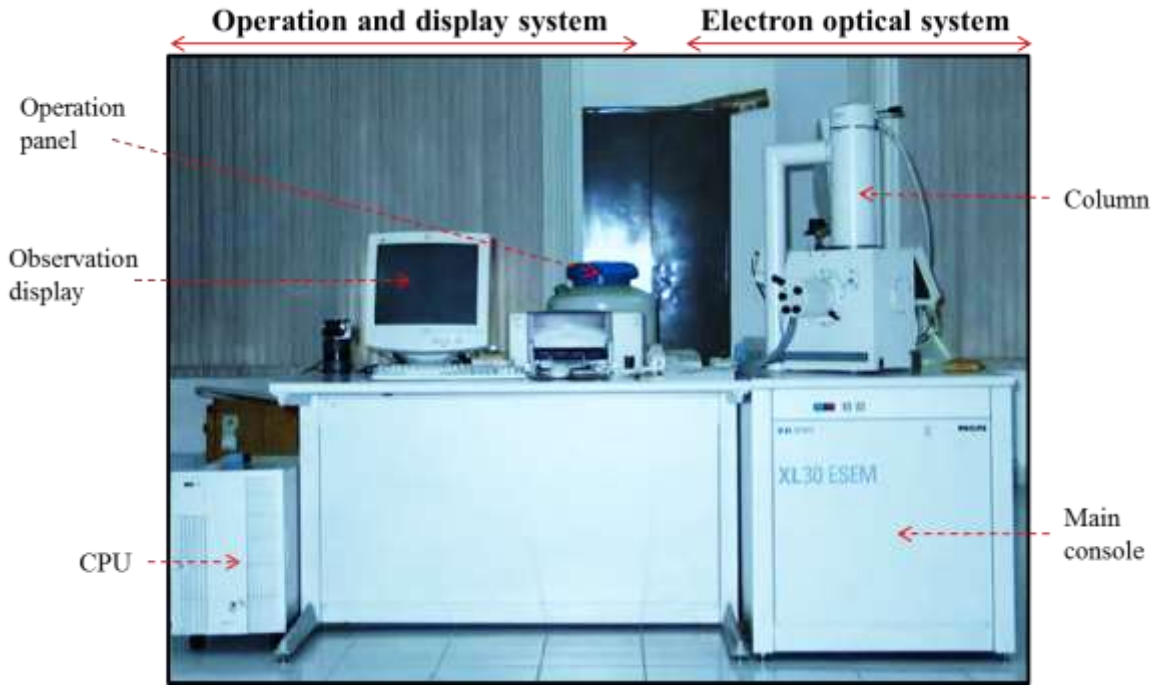
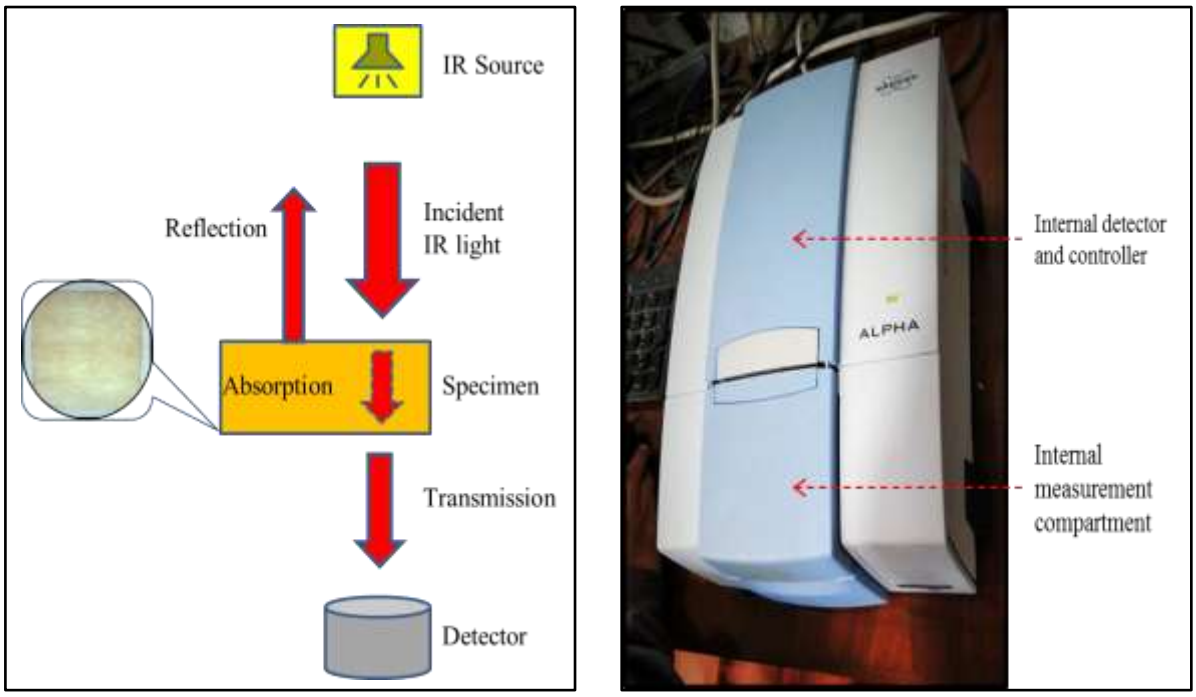


Figure 3.11: Environmental Scanning Electron Microscope

(Model: ESEM EDAX XL 30, Philips, Netherlands)



Principle of IR spectroscopy **Instrument**

Figure 3.13: FTIR spectrometer, model: BRUKER ALPHA

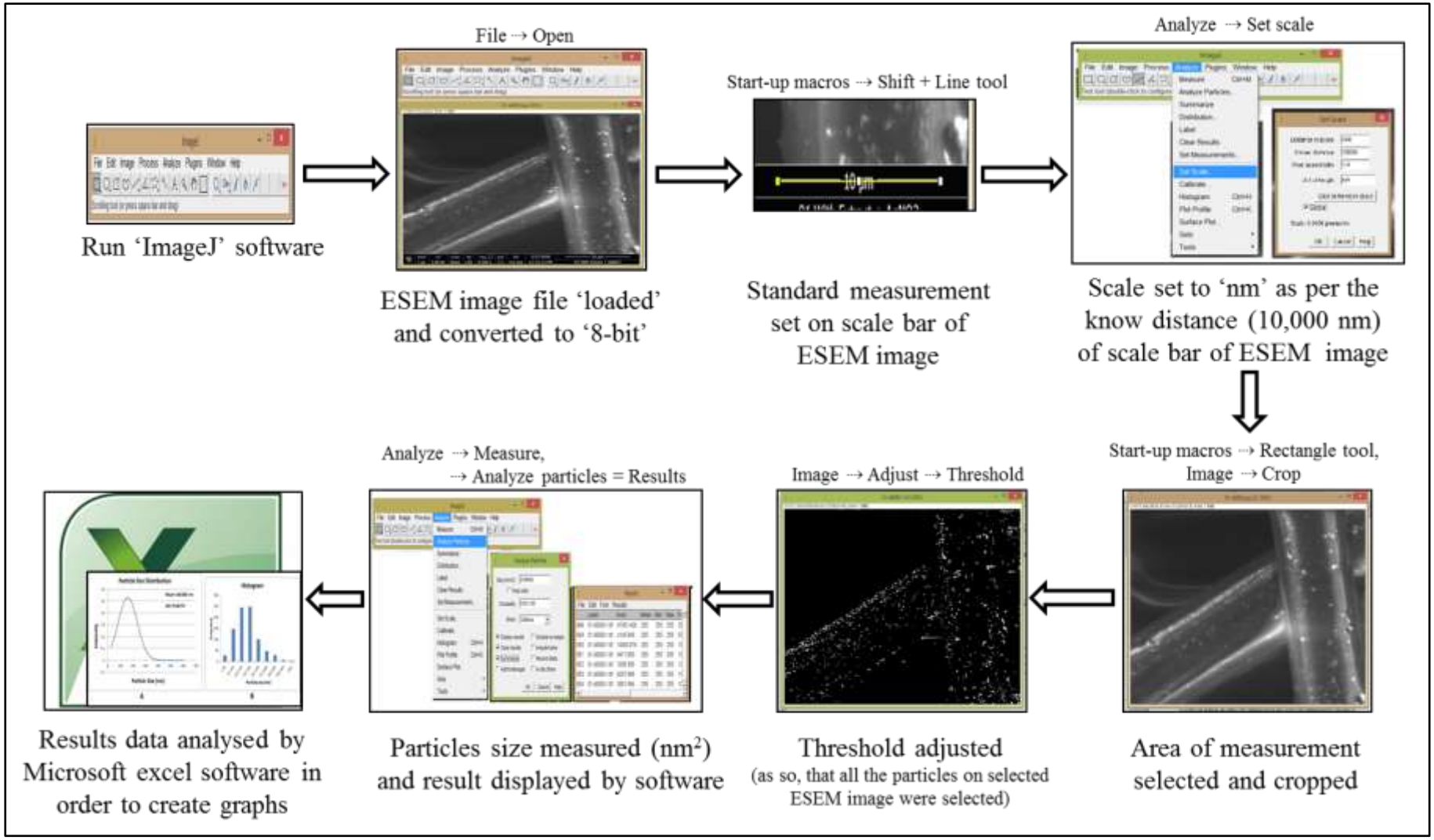


Figure 3.12: ESEM image processing by 'ImageJ' software

3.2.3.2.4 FTIR analysis

The chemical properties of the untreated PV non-woven (Samples 00) and PV-AgNPs/CP nano-composite (Samples 01) were evaluated using Fourier transform infrared spectroscopy (FTIR). FTIR spectrometer, model: BRUKER ALPHA (Figure 3.13) was used to record the FTIR spectra of the samples. Fabric/composite samples were positioned horizontally on the zinc selenide crystal and subjected to the infra-red light, crossing the sample from one end and exiting to the other end. Thus records automatically the FTIR spectra of the samples in the diffuse reflection mode at a resolution of 4 cm^{-1}. All the measurements were carried out in the range of 400 to 4,000 cm^{-1}.

3.2.3.3 Physical and comfort-related properties

3.2.3.3.1 Physical assessment

Influence of AgNPs treatment on imperative physical properties for bio-medical application, was evaluated in reference to base sample.

GSM (Gram per square meter): Fabric weight per unit area was measured in grams per square meter (GSM) by the gravimetric method according to ASTM D 3776-1996, IS1964-2001. A sample of 10×10cm was cut precisely with a sharp scissor after marking with the template. The sample weight (W) was measured on an electronic balance (Figure 3.14) with an accuracy of 0.01 gram and the GSM value was calculated as per equation 3.5.

$$\textbf{Fabric GSM} = \mathrm{W} \times 100 \quad \ldots\textbf{(3.5)}$$

Average of ten randomly selected samples for treated (t) and untreated (ut) fabrics was considered and their coefficient of variation (CV %) was calculated as follows:

$$\textbf{Coefficient of variation (cv)}\% = \frac{\text{standard deviation } (\sigma_{n-1})}{\text{Average } (\bar{X})} \times 100 \quad \ldots\textbf{(3.6)}$$

Where,

$$\textbf{Standard deviation } (\sigma_{n-1}) = \sqrt{\sum \frac{(X-\bar{X})^2}{(n-1)}} \quad \ldots\textbf{(3.7)}$$

x = observed values; $\bar{X}$ =mean values; n = no. of observations

Percent change in GSM was calculated by equation:

$$\boldsymbol{Change\ in\ GSM}(\%) = \frac{GSM_T - GSM_{UT}}{GSM_{UT}} \times 100$$

…**(3.8)**

Where,

'T' is treated and 'UT' is untreated fabrics

***Thickness*:** Thickness of both the fabrics/composites were measured according to ASTM D 1777-197, IS 7702-1975 standard at 0.1 lbs/inch^2 pressure using Mitutoya thickness gauge, Made in Japan (Figure 3.15). It works on the principle of determination of thickness of a compressible material and consists of precise measurement of vertical distance between two parallel round plates, separated by a cloth or fabric thickness under a known arbitrary applied pressure. The instrument consists of two parallel round plates. Top smaller plate is known as pressure foot and the other larger one is anvil. The clock type of dial gauge is provided which has the capacity of measuring 0.01mm – 10mm thickness. The Pressure foot and anvil were cleaned with the help of clean paper and dial of the gauge was checked for zero position. Fabric sample was placed on the anvil and the pressure foot was brought down slowly in contact with the fabric under test at the desired pressure (0.1 lbs/sq. inch). The thickness was read from the dial of the instrument when the movement of pointer has stopped. Average and CV% [Equations 3.6 and 3.7 respectively] of ten randomly selected positions were calculated.

Percent change in thickness was calculated by equation…

$$\boldsymbol{Change\ in\ Thichness}(\%) = \frac{Th_T - Th_{UT}}{Th_{UT}} \times 100$$

…**(3.9)**

Where,

Th_T = Thickness of Treated fabric

Th_{UT} = Thickness of Untreated fabric

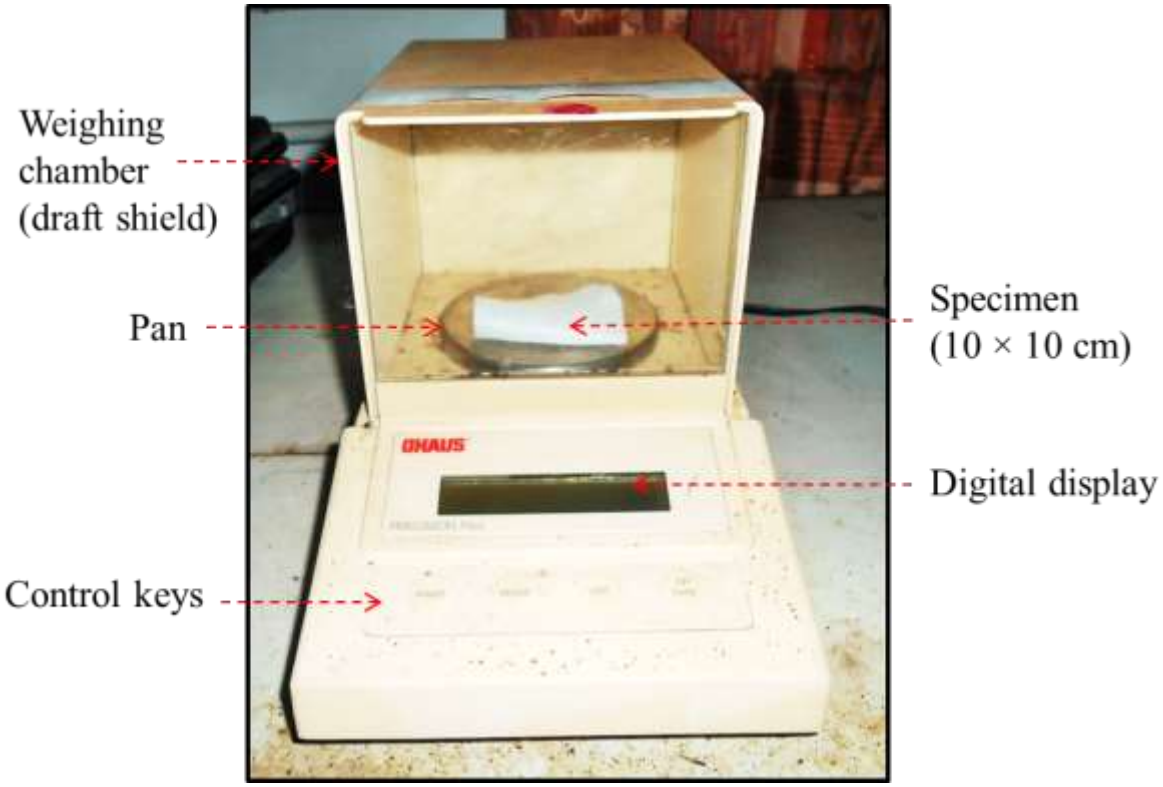


Figure 3.14: Weighing Scale (Electronic balance)

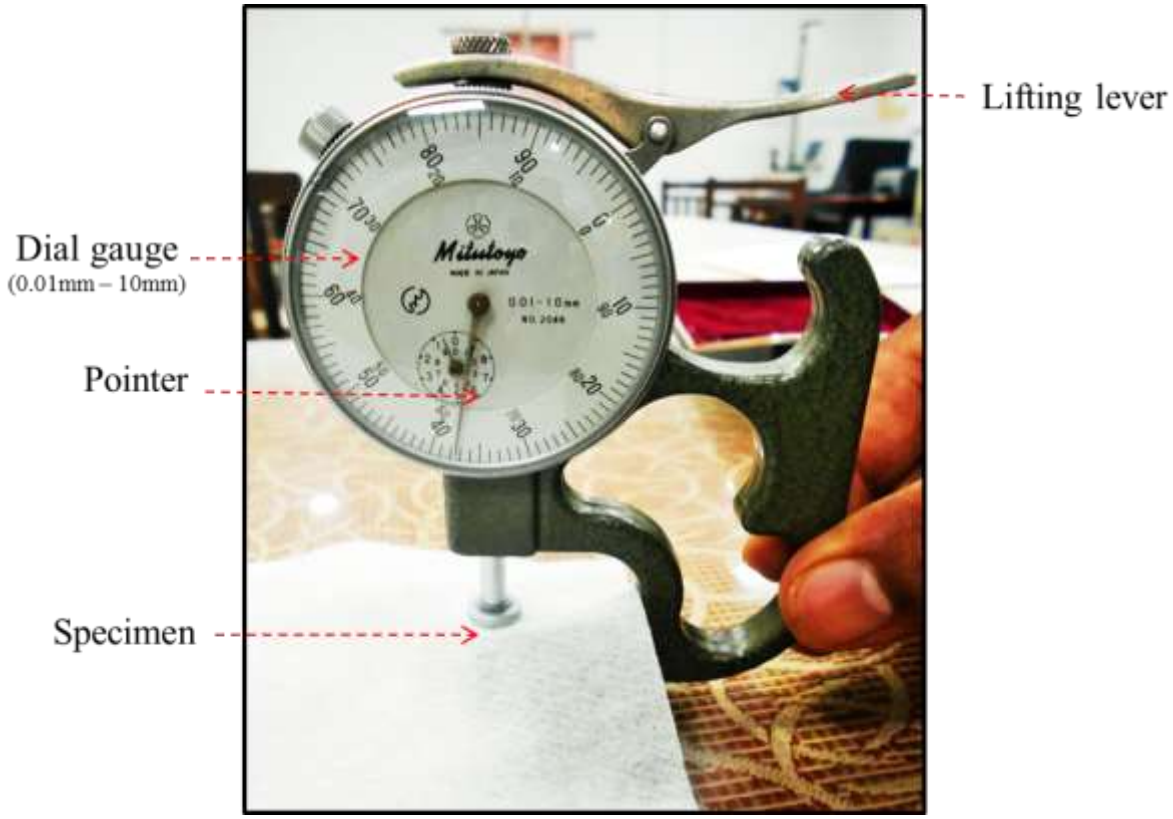


Figure 3.15: Fabric thickness gauge

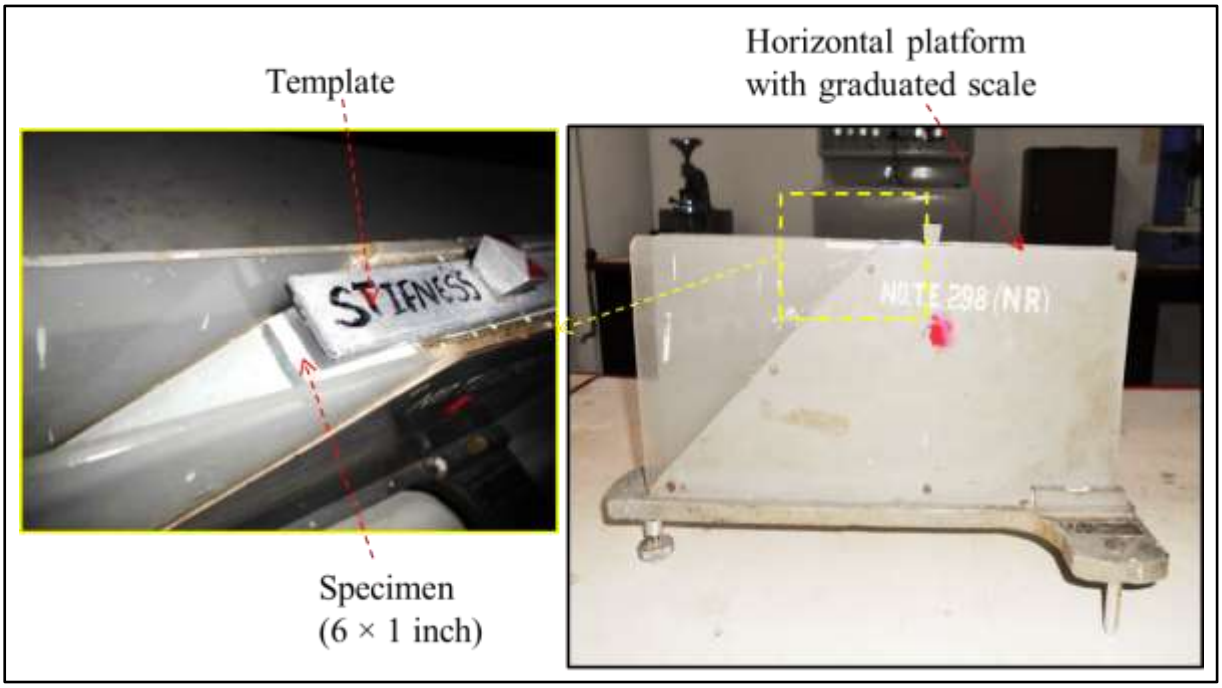


Figure 3.16: Shirley stiffness tester

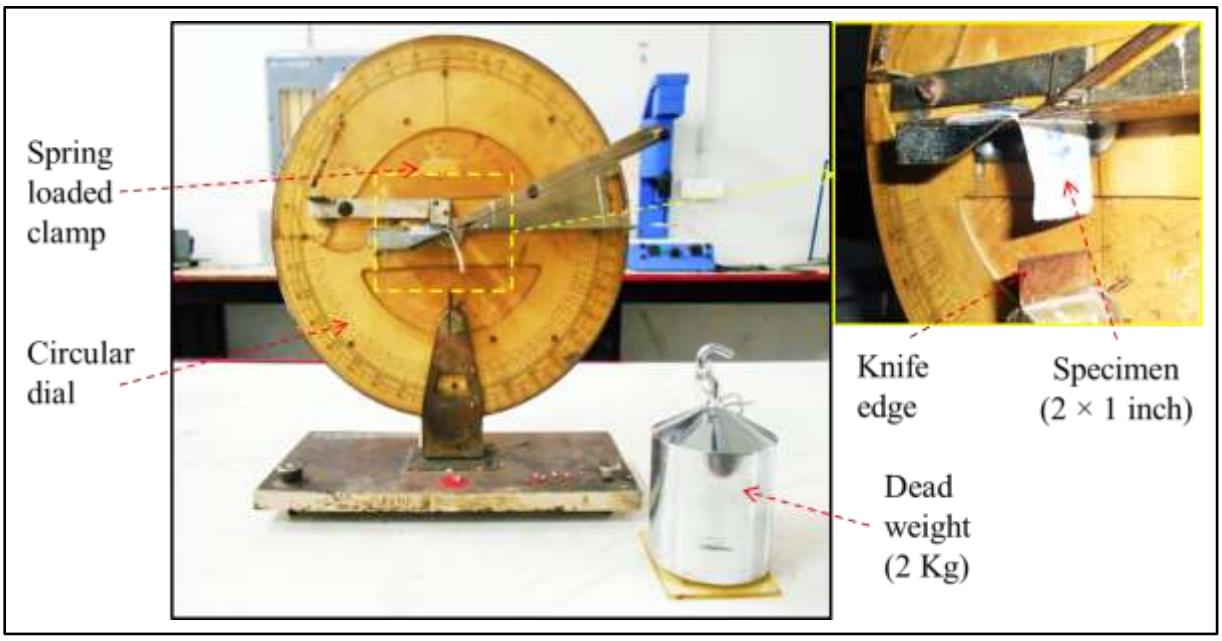


Figure 3.17: Fabric crease recovery tester

3.2.3.3.2 Low-stress properties

Bending modulus and crease recovery are two important low-stress properties likely to affect performance of bio-medical material. Thereby change in their values on the application of the functional nanoparticles was evaluated.

Bending modulus: Stiffness is the property of fabric that indicates its resistance to bend or drape. A stiffness test (ASTM D1388) was carried out on Shirley stiffness tester (Figure 3.16). A rectangular strip of 6×1 inch was cut from the fabric marked precisely with the help of a template. By placing fabric below the template, both template and the sample were transferred together onto the horizontal platform. Both were slowly pushed forward in such a way that fabric strip overhangs like a cantilever and bends downwards over the edge of the platform at a bending angle of 41.5º. Movement of template and fabric was continued until the tip of specimen touched the index line. The straight length (l) of fabric at the point of bending was read-out from the scale graduated in centimetre, engraved on the side edges of the platform. An average of ten samples was considered to calculate bending length(c), flexural rigidity (G) and bending modulus (q) of the treated and untreated specimen at their measured weight (W, in g/cm^2) and thickness (g, in cm) [Equations 3.10 – 3.12].

$$\textbf{Bending length c(cm)} = \text{l in cm} \times 0.5 \quad \ldots\textbf{(3.10)}$$

$$\textbf{Flexural Rigidity (G)} = \text{W} \times \text{C}^3 \times 10^3 \text{ mg. cm} \quad \ldots\textbf{(3.11)}$$

$$\textbf{Bending Modulus (q)} \left(\frac{\boldsymbol{Kg}}{\boldsymbol{cm^2}}\right) = \frac{12\ \text{G} \times 10^{-6}}{(g)^3} \quad \ldots\textbf{(3.12)}$$

Change in bending modulus on AgNPs treatment was expressed as a percentage of untreated sample's bending modulus.

Crease recovery angle: Shirley crease recovery tester (Figure 3.17) was used to determine crease recovery of both the samples as per AATCC 66-2003 standard. It determines ability of fabric undergone the deformation to recover from crease or wrinkle in terms of crease recovery angle (Θ). The specimen of 2×1 inch size was cut by using the template and carefully folding in half. The folded specimen was sandwiched between two glass slides and kept for crease development under specified load of 2 kg for 60 seconds. The creased sample was then clamped on the tester in such a way that one fold was gripped by spring-loaded clamp leaving second half fold to fall free under its own weight. The clamped sample was allowed to recover from the crease and on full recovery (no further change noticed in its free end position); the dial of the instrument was rotated till the free edge of the specimen came in line with the knife edge. The recovery angle was read from the engraved scale through reading glass window. Three tests per sample were done and their mean value was considered in calculating percent change in crease recovery on treatment.

3.2.3.3.3 Comfort-associated properties

Comfort of the wearer is the prime consideration for any type of bio-medical textile material. Air permeability and Overall moisture management capability (OMMC) of the newly engineered PV-AgNPs/CP nano-composite and the untreated base material were thereby measured to determine influence of treatment on air and moisture transportation efficacy.

Air permeability: Air permeability refers, unit volume of the air passes through unit area of the fabric in unit time at 10 mm water level pressure and expressed in $cm^3/cm^2/sec$ or $m^3/m^2/hr$ (ASTM D737) [255]. It was measured using METEFEM Air Permeability Tester (Figure 3.18). The measurements were performed at a constant pressure drop of 100 Pa (5.04 cm^2 test area). The specimen was clamped in the test rig of one inch diameter and the volume of air in cubic meter passed per hour through one square meter of the fabric at a constant pressure of 10mm of water level was read directly from interchangeable test head (Rotameter) of the instrument. Average of ten air permeability values measured from randomly selected positions for each sample was taken into the consideration. Change in air permeability value on AgNPs treatment was expressed as a percentage of the untreated sample's air permeability.

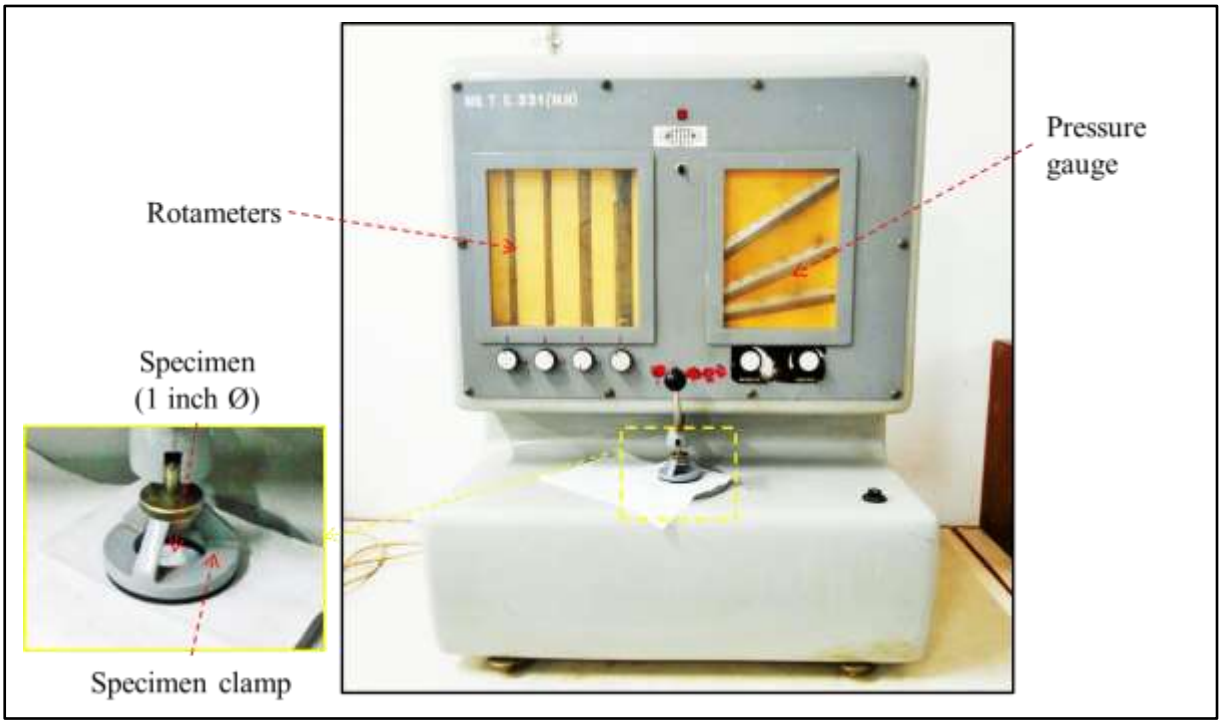


Figure 3.18: Air Permeability Tester

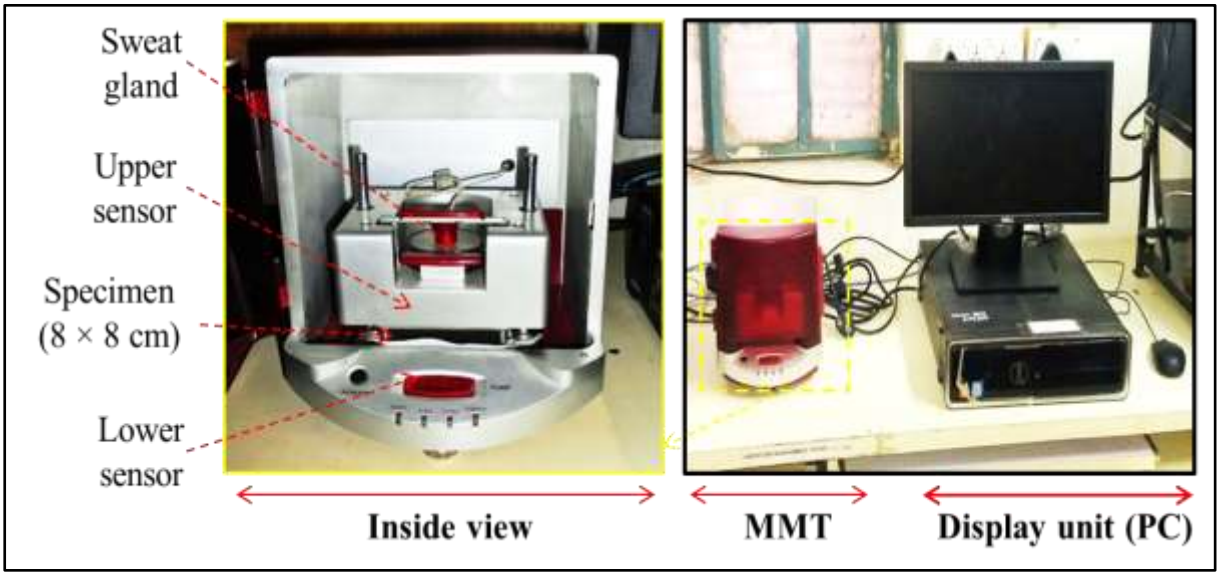


Figure 3.19: SDL ATLAS Moisture Management Tester

Overall moisture management capability (OMMC): This test was done to define the liquid moisture management capability of the fabric relating perspiration management of the wearer as per AATCC-195 [289]. The SDL-ATLAS MMT (Figure 3.19) designed to sense, measure, and record the liquid moisture transport behaviour in multiple directions was used for this purpose. The test specimen was cut by using a template of 8×8 cm and conditioned before subjecting for the MMT. The instrument has two; upper and lower concentric moisture sensors between which the test sample was placed. Before starting the test, time of measurement was set for 120 seconds. A pre-defined amount (~0.22 cc) of test solution representative of human perspiration made by dissolving 9 g NaCl in 1 liter DDW having 16 ± 2 mS (milli Siemens) electrical conductivity was put onto the upper surface (skin side) of the fabric and allowed to transfer in three directions onto the material, viz;

i) Radial spreading on the upper surface of the fabric.
ii) Transferring through the fabric from the upper surface to the bottom surface
iii) Radial spreading on the lower surface of the fabric.

The liquid moisture management characterization of the test specimen is based on its electrical resistance changes. Various MMT indices (Table 3.4) were calculated by the interfaced microprocessor software.

Table 3.3: Grading specification of MMT test as per AATCC 195

Parameter	Surface	Grade				
		1	2	3	4	5
Wetting time, (s)	Top	> = 120 No wetting	20 – 119 Slow	5 – 19 Medium	3 – 5 Fast	<3 Very fast
	Bottom	> = 120 No wetting	20 – 119 Slow	5 – 19 Medium	3 – 5 Fast	<3 Very fast
Absorption rate (%/s)	Top	0 – 10 Very slow	10 – 30 Slow	30 – 50 Medium	50 – 100 Fast	>100 Very fast
	Bottom	0 – 10 Very slow	10 – 30 Slow	30 – 50 Medium	50 – 100 Fast	>100 Very fast
Max. wetted radius, (mm)	Top	0 – 7 No wetting	7 – 12 Small	12 – 17 Medium	17 – 22 Fast	>22 Very fast
	Bottom	0 – 7 No wetting	7 – 12 Small	12 – 17 Medium	17 – 22 Fast	>22 Very fast
Spreading speed, (mm/s)	Top	0 – 1 Very slow	1 – 2 Slow	2 – 3 Medium	3 – 4 Fast	>4 Very fast
	Bottom	0 – 1 Very slow	1 – 2 Slow	2 – 3 Medium	3 – 4 Fast	>4 Very fast
AOTI	-	< -50 Very poor	-50 – 100 Poor	100 – 200 Good	200 – 400 Very good	>400 Excellent
OMMC	-	0 – 0.2 Very poor	0.2 – 0.4 Poor	0.4 – 0.6 Good	0.6 – 0.8 Very good	>0.8 Excellent

The software has also illustrated MMT performance of the tested specimen graphically along with grading as per test specification given in Table 3.3.

Table 3.4: Indices of MMT

Sr. no.	Indices	Interpretation
1.	**Wetting time (s)** – (WTt), and (WTb)	The time in seconds (s) when the top (t) and bottom (b) surfaces of the specimen begin to be wetted after the test is started
2.	**Absorption rate (%/s)** – (ARt), and (ARb)	The average speed of the liquid moisture absorption for the top and bottom surfaces of the specimen during the initial change of water content during a test.
3.	**Max. wetted radius (mm)** – (MWRt), and (MWRb)	The greatest radius of water ring measured on the top and bottom surfaces.
4.	**Spreading speed (mm/s)** – (SSt), and (SSb)	The rate of top and bottom surface wetting from the center of the specimen where the test solution is dropped to the maximum wetted radius
5.	**Accumulative one-way transport index (AOTI)**	The difference between the area of the liquid moisture content curves of the top and bottom surfaces of a specimen with respect to time
6.	**Overall (liquid) moisture management capability (OMMC)**	An index of the overall capability of a fabric to transport liquid moisture as calculated by combining three measured attributes of performance: the liquid moisture absorption rate on the bottom surface (ARb), accumulative one way transport index (AOTI), and the maximum liquid moisture spreading speed on the bottom surface (SSb). OMMC = 0.25 ARb + 0.5AOTI + 0.25 SSb
** t = top surface, and b= bottom surface*		

3.2.3.4 UV Transmission properties

The Ultraviolet Protection Factor (UPF) of the untreated PV-nonwoven (sample 00) and PV-AgNPs/CP nano-composite (sample 01) were recorded by using an Ultraviolet Transmittance Analyser UV-2000F, Labsphere (Figure 3.20) according to EN 13758-1:2002 standard [241]. This Ultraviolet transmittance analyzer incorporates the latest and highly application specific ultraviolet spectroscopy. The instrument is rapidly operated by measuring the diffuse transmittance of textile materials as a function of wavelength in the ultraviolet spectral region from 250 nm to 450 nm. It also ensures the automatic calculations of spectral transmittance, UPF, critical wavelength, and UVA : UVB ratios of the textile materials.

The specimen to be tested was placed at the transmission port of the integrating sphere for measuring its UV transmission value with the help of Xenon flash lamp. The location for the testing position from 1 to 9 can be set with the help of moving sample positioning stage. Each sample was tested for five different locations and different UV transmission profiles were calculated. The measuring area for the individual location was 0.67 cm^2 and wavelength accuracy was ±1 nm with dynamic range extension up to 2.7 Å (Angstrom).

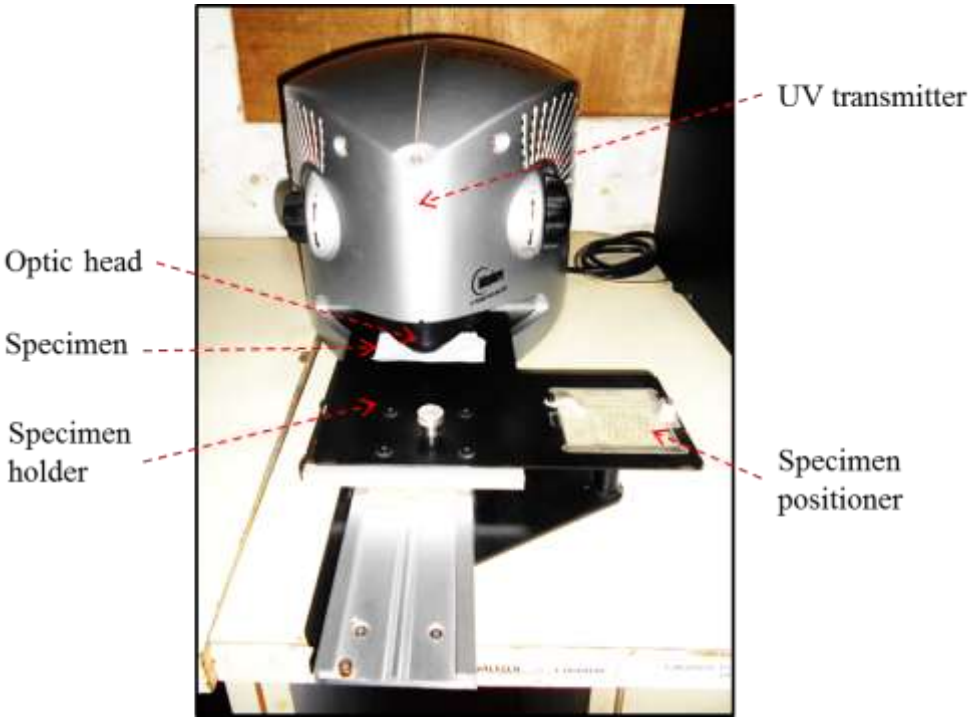


Figure 3.20: The Ultraviolet Transmittance Analyser UV-2000F

3.3 PHASE–III:

OPTIMIZATION OF AgNPs/CP COLLOIDAL CONSTITUENTS' ADD-ON FOR ANTIBACTERIAL ACTIVITIES IN NONWOVEN @PV-AgNPs/CP NANO-COMPOSITES

Aim: This phase was designed with following objectives:

I. Optimizing silver nitrate ($AgNO_3$) salt add-on and CP leave extract concentration in newly designed colloidal green synthesis process and analyzing its antibacterial efficacy into prototype bio-medical nonwoven nano-composite.
II. Preparation of prototype composites by treating the base nonwoven fabric only with selected concentrations of CP leave extract and evaluates their antibacterial performance with respect to equivalent AgNPs/CP treated nano-composites.

3.3.1 MATERIALS

Materials used in this phase of experiments were same as in phases (I and II), except their concentration levels.

i) **Silver Nitrate salt ($AgNO_3$):** The concentrations used were ranging from 1000mM to 100mM, and 100mM to 0mM (nil) in major scale and minor scale experiments respectively.

ii) **CP leave extract:** The CP leave extract is a well-established traditional natural antibacterial agent, and it has been also validated in previous phase of the study. Thereby, its concentration for optimum antibacterial activities needs to be worked out. Experiments were conducted to create antibacterial medium in the presence and absence of $AgNO_3$ in the composite by varying its concentration from 5% to 20% with a constant 5% increment.

iii) **Base textile material (Fabric):** Popularly used PV-nonwoven fabric was continued to be used as bio-medical base material in this phase also to create intended composite samples.

iv) **Antibacterial Test materials:** Nutrient broth, Agar- Agar powder and DDW used in this phase of study were having the same specifications as mentioned in phase II except change in the test method and accordingly their utilization.

3.3.2 METHODS

The desired AgNPs/CP colloidal were synthesized at laboratory scale by treating $AgNO_3$ salt with CP leave extract in the same manner as described in phase I. Effect of varying concentration of both the participating constituents on stability of the colloidal, particle size and antibacterial activities were analysed by conducting pilot trials spread into two groups, viz; i) Major scale, and ii) Minor scale.

3.3.2.1 MAJOR SCALE TRAILS

This set of trail was worked on optimization of the $AgNO_3$ salt by its major scale addition; here scale pertained to the molarities of the $AgNO_3$ solution. The molarities used for treating salt solution with CP leave extract were 1000 mM, 500 mM, 200 mM, and 100 mM respectively. Four CP leave extracts concentrations, i.e. 05%, 10%, 15%, and 20% were used during colloidal synthesis trials. Selection of minimum level for each component was persisted by the previous research done on green synthesis of $AgNO_3$ but with different natural mediums [118,181,183,190,194,235,290]. However in case of the salt, 100mM has also shown very poor stability with the other natural mediums when green synthesized. Thereby, in the present study major scale selection from minimum level onwards was broaden to analyse the impact of too high add-on of costlier $AgNO_3$ on the AgNPs stability with reduced number of samples as suggested in back stuff record [6,79,131,179,237]. The required concentration of $AgNO_3$ salt solutions and CP leave extracts were prepared in the same way as described in phase I, only the changes made were in their add-on values (Tables 3.5 and 3.6).

Table 3.5: Composition details for CP leave extracts

Sr. No.	Extracts code	% Concentration of the Extract	Amount of CP leave powder (g)	Amount of DDW (ml)
1.	E05	05 %	50 g	1000 ml
2.	E10	10 %	100 g	1000 ml
3.	E15	15 %	150 g	1000 ml
4.	E20	20 %	200 g	1000 ml

Table 3.6: Composition details for $AgNO_3$ salt solutions

Sr. No.	$AgNO_3$ Solution code	Molarity of $AgNO_3$ (mM)	Amount of the $AgNO_3$ (g)	Amount of the DDW (ml)
		Major scale		
1.	S01	100 mM	0.169 g	10 ml
2.	S02	200 mM	0.339 g	10 ml
3.	S05	500 mM	0.849 g	10 ml
4.	S10	1000 mM	1.698 g	10 ml
		Minor scale		
		Set–I		
1.	S0020	20 mM	0.0340 g	10 ml
2.	S0040	40 mM	0.0679 g	10 ml
3.	S0060	60 mM	0.1019 g	10 ml
4.	S0080	80 mM	0.1359 g	10 ml
5.	S0100	100 mM	0.1698 g	10 ml
		Set–II		
6.	S0001	01 mM	0.0017 g	10 ml
7.	S0005	05 mM	0.0085 g	10 ml
8.	S0010	10 mM	0.0169 g	10 ml
9.	S0015	15 mM	0.0254 g	10 ml

Table 3.7: Composition used for AgNPs/CP colloidal solutions (Major scale)

Sr. No.	AgNPs/CP composites Set No.	AgNPs/CP colloidal solution (Code)	% Con. of the Extract	Molarity of $AgNO_3$ (mM)
1.		A1	E05S00	5%	---
2.		A2	E05S01	5%	100
3.	A	A3	E05S02	5%	200
4.		A4	E05S05	5%	500
5.		A5	E05S10	5%	1000
6.		B1	E10S00	10%	---
7.		B2	E10S01	10%	100
8.	B	B3	E10S02	10%	200
9.		B4	E10S05	10%	500
10.		B5	E10S10	10%	1000
11.		C1	E15S00	15%	---
12.		C2	E15S01	15%	100
13.	C	C3	E15S02	15%	200
14.		C4	E15S05	15%	500
15.		C5	E15S10	15%	1000
16.		D1	E20S00	20%	---
17.		D2	E20S01	20%	100
18.	D	D3	E20S02	20%	200
19.		D4	E20S05	20%	500
20.		D5	E20S10	20%	1000

** --- = Not used*

Colloidal synthesis was done by treating each major scale 10 ml of $AgNO_3$ salt solution with 100 ml of the four selected CP leave extract concentrations, i.e. 05%, 10%, 15%, and 20%. Thus total four sets of trials; nomenclated as A, B, C and D respectively were conducted and each category was having five combinations (Table 3.7). The other particulars of the AgNPs/CP colloidal preparation were same as followed in case of the reverse green synthesis method (bottom-up approach) mentioned in phase-I.

3.3.2.1.1 Testing and Analysis

All the prepared AgNPs/CP colloidal solutions were kept for colour change observation, preliminary stability test, before applying on PV-nonwoven fabric to prepare prototype PV-AgNPs/CP nano-composites. The samples were then analyzed for their antibacterial activity.

3.3.2.2 MINOR SCALE TRAILS

According to the antibacterial activity test results of major scale trials; sample 'E10S01' has shown the best performance against both the bacterial cultures i.e. Staphylococcus aureus and Escherichia coli. Even it was observed during preliminary visual judgment colloidal stability testing; samples produced on treatment with 100 mM concentrated $AgNO_3$ solutions (S01) have executed better stability invariably for all the groups. However, this preferable state was lasted only for first 24 hours of observation and later on occupied non-preferable blackish tint during 24 to 48 hours observation slot. This change is indicative of formation of macromolecules on clustering of nanoparticles. Thereby second set of pilot trials were designed to carry out colloidal synthesis by scaling down (<100 mM) the molarities of $AgNO_3$ and to work out the best possible level. The minor scale trials were conducted in first set with 100mM, 80 mM, 60 mM, 40mM and 20mM molarities (Table 3.6) of $AgNO_3$ by keeping the concentration of the CP leave extract to 10% (E10) only (Table 3.5). This selection of optimum CP leave extract concentration was validated by better antibacterial activities (highest average zone of inhibitions) observed in all categories of major scale trials. The outputted colloidal of this part's minor scale trials were then subjected for preliminary visual stabilization test and antibacterial activities.

The best results in terms of colloidal colour and its stability as well as antibacterial activities were found with sample 'E10S0020' produced with 20mM $AgNO_3$ concentration. Thereby second set of minor scale trials were conducted to precise the salt solution's

minimum viable molarity for optimized antibacterial activities of the synthesized stable colloidal. In these trials molarity level of $AgNO_3$ was further scale down from 20mM with smaller decrement; 15mM, 10mM, 5mM and 1mM, and colloidal were produced with 10ml $AgNO_3$ salt solution in combination with 100ml of 10% CP extract for each interaction (Table 3.6). Thus total two sets of trials; nomenclated as E and F respectively were conducted and each category were having five combinations (Table 3.8). These trials have facilitated in identifying optimized molarity of $AgNO_3$ salt.

Table 3.8: Composition used for AgNPs/CP colloidal solutions (Minor scale)

Sr. No.	PV-AgNPs/CP composites Set No.		AgNPs/CP colloidal solution (Code)	% Con. of the Extract	Molarity of $AgNO_3$ (mM)
			Set–I		
1.		E1	E10S0020	10%	20
2.		E2	E10S0040	10%	40
3.	E	E3	E10S0060	10%	60
4.		E4	E10S0080	10%	80
5.		E5	E10S0100	10%	100
			Set–II		
6.		F1	E10S0000	10%	---
7.		F2	E10S0001	10%	1
8.	F	F3	E10S0005	10%	5
9.		F4	E10S0010	10%	10
10.		F5	E10S0015	10%	15

** --- = Not used*

3.3.2.2.1 Testing and Analysis

All the colloidal were evaluated invariably for AgNPs formation confirmation and their stability as well as antibacterial assessment in this course of study. However, the samples at the end of these analyses found promising were only considered for further evaluations; UV spectrum, Particle size analysis. In addition to this MTT assay and GC-MS analysis were also used for cytotoxicity and chemical compositions respectively. The overall test results were pondered for identifying the optimum sample.

A) Visual colour change observation (VCCO) of the AgNPs colloidal

Nanoparticles formation in the prepared colloidal solutions has given preliminary confirmation via visual or colour change observation in similar fashion as done in phase – I (Section 3.1.3.1). A visual colour judgment based creep studies for 0 hour, 24 hours, 48 hours, 1 week, 1 month and 6 months were also conducted to determine extent of AgNPs stability in the colloidal solutions.

B) Antibacterial assessment by disc diffusion method (SN 195 920 standard)

Hence number of samples in consideration are more in number; thirty (major: 20 and minor: 10) in this phase of study, thereby SN 195 920 standard method has been followed instead of AATCC 147 used for two samples in phase II. This method has relatively simple and ability to test easily multiple antibacterial agents on each bacterial isolates (Petri plate) [268]. The test procedure includes following steps:

i. Preparation of PV-AgNPs/CP nano-composite discs and
ii. Antibacterial assessment

i) Preparation of PV-AgNPs/CP nano-composite discs

The selected base material; PV-nonwoven was cut into fixed fabric swatches of 1×1 inch size. These swatches were treated with respective colloidal solution by cold dipping technique and dried at the room temperature (Figure 3.21). Such prepared PV-AgNPs/CP nano-composites were then cut into test specific 5mm circular sample discs for the antibacterial assessment.

ii) Antibacterial assessment

The antibacterial activity was checked against both gram positive and gram negative types of bacterial cultures viz., Staphylococcus aureus (NCIM 2654) and Escherichia coli (NCIM 2832) [2,3,265] as per disc diffusion method (SN 195 920 standard) [268]. The steps followed during testing are listed below and illustrated graphically in Figure 3.22.

a. Preparation of required bacterial culture medium
    1. Preparation of nutrient agar media
    2. Preparation of petri dishes for testing
    3. Preparation and maintenance of bacterial culture medium
b. Loading of the bacterial test cultures into duly labeled Petri dishes, and
c. Antibacterial assessment.

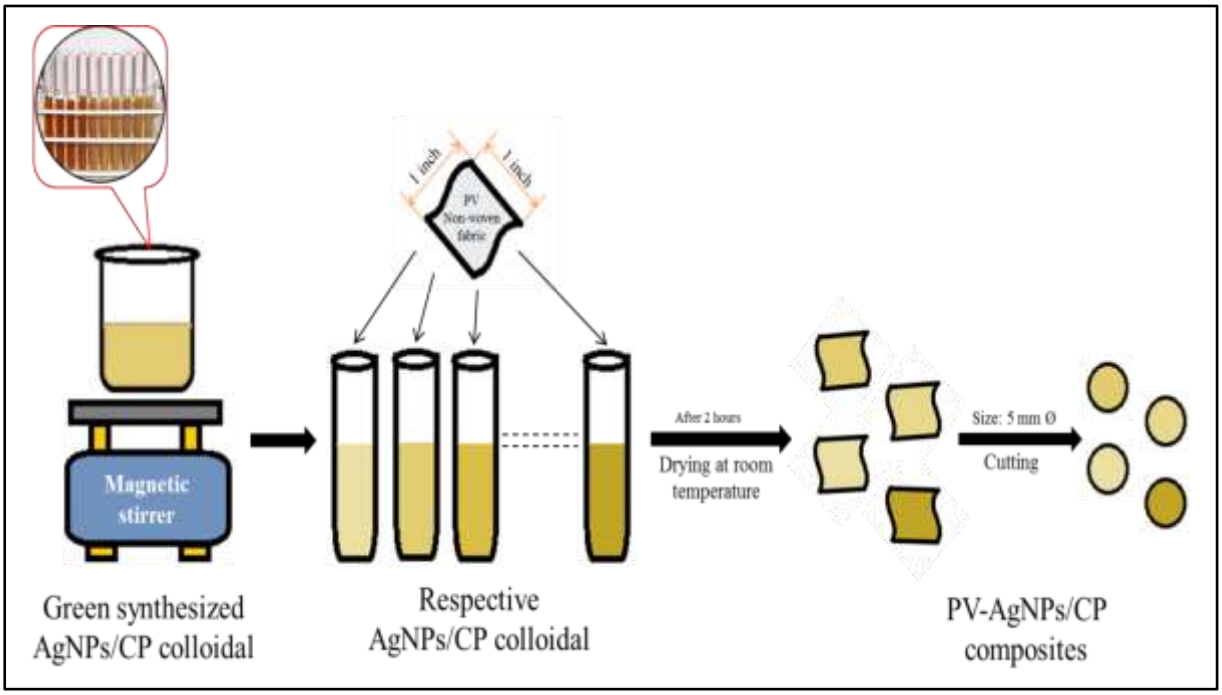


Figure 3.21: Preparation of the PV-AgNPs/CP composites

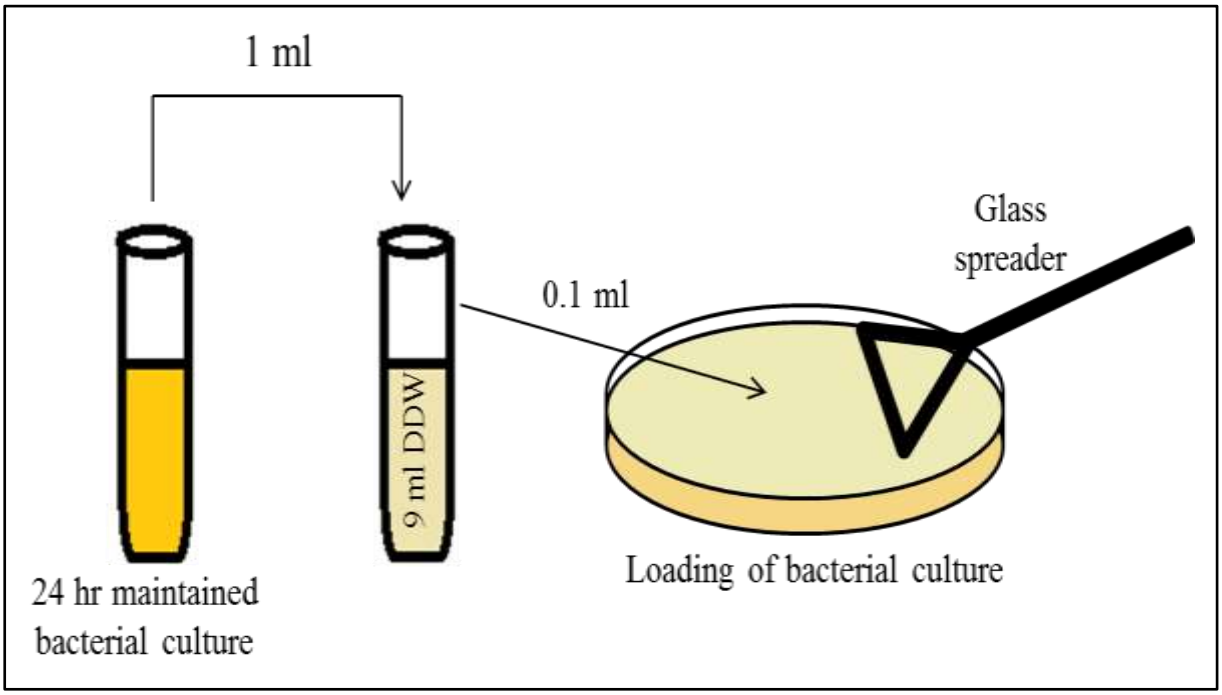


Figure 3.23: Transferring and spreading of bacterial culture

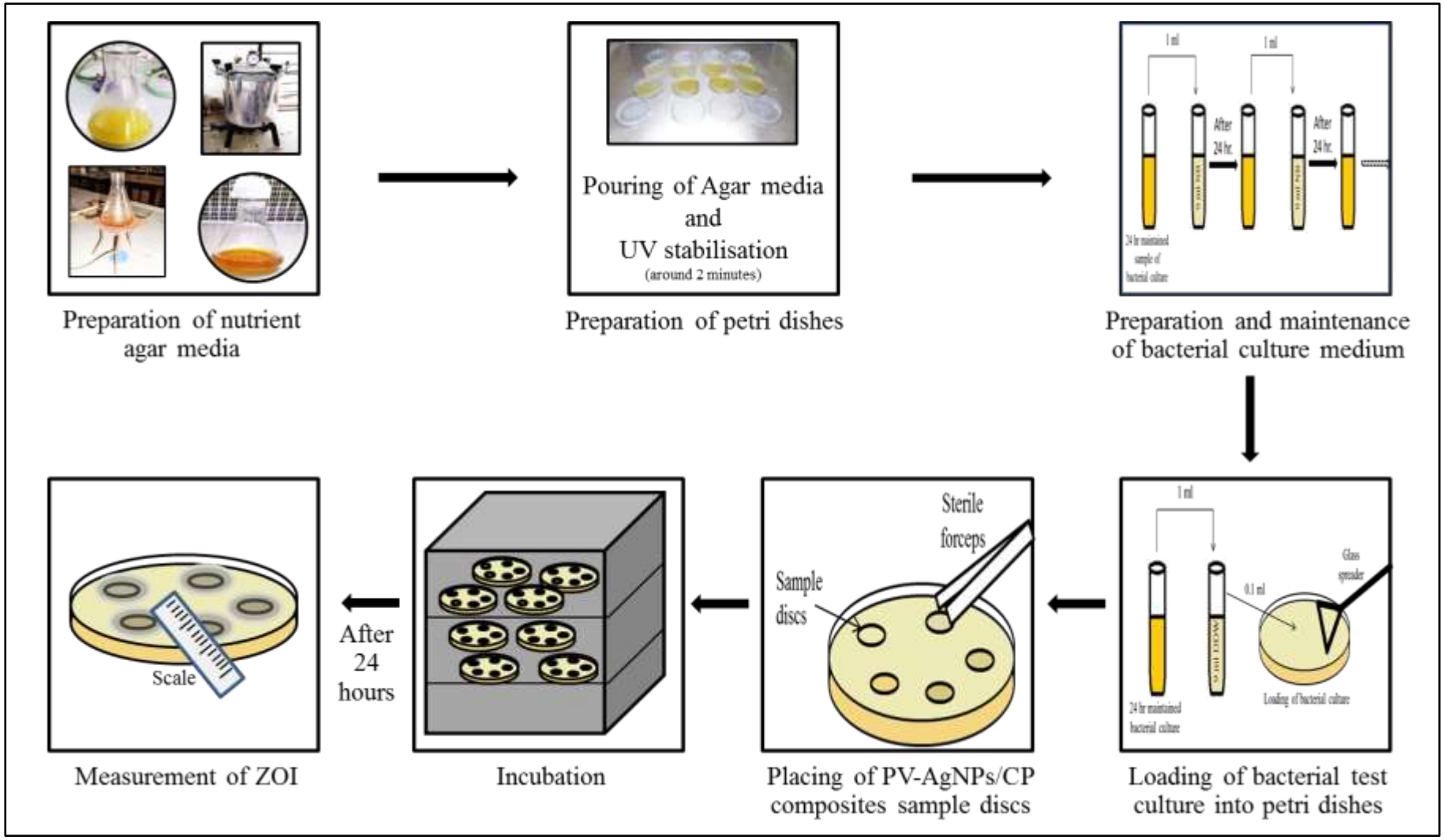
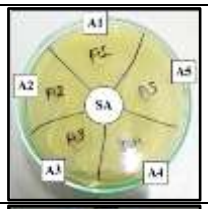
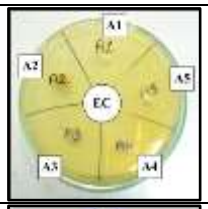
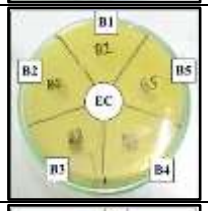
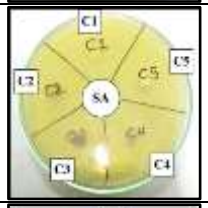
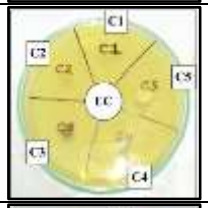
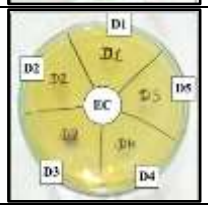
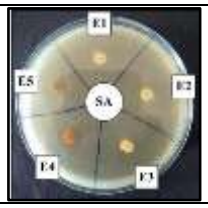
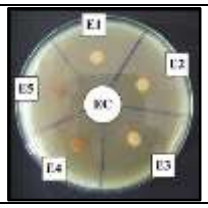
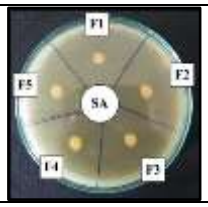
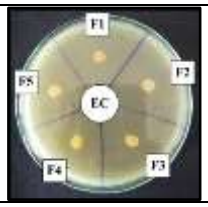


Figure 3.22: Antibacterial assessment by disc diffusion method (SN 195 920 standard)

Table 3.9: Antibacterial test samples details

Sample code	Colloidal solution (Code)	Bacterial culture	
		Staphylococcus aureus (SA)	Escherichia coli (EC)
Major scale			
A1	E05S00©		
A2	E05S01		
A3	E05S02		
A4	E05S05		
A5	E05S10		
B1	E10S00©		
B2	E10S01		
B3	E10S02		
B4	E10S05		
B5	E10S10		
C1	E15S00©		
C2	E15S01		
C3	E15S02		
C4	E15S05		
C5	E15S10		
D1	E20S00©		
D2	E20S01		
D3	E20S02		
D4	E20S05		
D5	E20S10		
Minor scale			
Set–I			
E1	E10S0020		
E2	E10S0040		
E3	E10S0060		
E4	E10S0080		
E5	E10S0100		
Set–II			
F1	E10S0000©		
F2	E10S0001		
F3	E10S0005		
F4	E10S0010		
F5	E10S0015		

© = *Control sample*

a) Preparation of required bacterial culture medium

The methodologies adopted in the preparation of required bacterial culture medium for all the three steps (i–iii) were exactly similar to AATCC 147 method, used in phase II (Section 3.2.3.1). Thereby details are not repeated and the method is described from that point onwards here; i.e. it differed from the previously adopted one.

b) Loading of bacterial test culture into Petri dishes

The diluted bacterial inoculum (1.0 ± 0.1 ml of 24 hours maintained bacterial culture prepared into 9.0 ± 0.1 ml of sterile DDW with appropriate agitation) of 100 µl (0.1 ml) was transferred and spread uniformly on the surface of UV stabilised agar Petri plates with a L-shaped glass spreader (Figure 3.23). Precaution was taken not to break/damage the agar surface while transferring and spreading the bacterial culture.

Each Petri dish has been labelled as per the type of bacterial culture and test sample going to be loaded (Table 3.9).

c) Antibacterial assessment

The PV-AgNPs/CP nano-composites sample discs were then placed in the respective bacterial culture loaded agar plates with the help of sterile forceps. The samples in which $AgNO_3$ was not used i.e. only CP leave extract treated samples (A1, B1, C1, and D1 in major scale, and F1 in Minor scale) were considered as control samples, in order to evaluate only CP leave extract contribution in antibacterial activity at the selected concentration levels. Subsequently, all the plates were incubated at 37°C for 24 hours to check for the bacterial inhibition. After 24 hours of incubation, a clear area without bacteria; ZOI (Zone of Inhibition) was measured in mm with scale for each sample. Apart from this stability of antibacterial efficacy for time creep of 24 hours, 48 hours, and 72 hours was analysed for all the samples.

The average ZOI and percent change with time creep for the tested PV-AgNPs/CP nano-composites samples were calculated [Equations 3.13 and 3.14 respectively]. These values were used for comparison and determine the best sample in terms of efficacy and stability of the antibacterial treatment.

$$\textbf{Average width of ZOI in mm} = \frac{\sum_{i=1}^{n} Z_i}{n} \quad \ldots\textbf{(3.13)}$$

Where;
Z_i= ZOI in mm at the selected level of the participating component,

$$\textbf{Percent Change in ZOI} = \frac{Z_a - Z_b}{Z_a} \times 100 \quad \ldots\textbf{(3.14)}$$

Where;
Z_a= ZOI in mm at the beginning of the test,
Z_b= ZOI in mm after respective time creep

C) UV-VIS Spectroscopy of AgNPs/CP colloidal solutions

Ultraviolet-Visible (UV-VIS) spectroscopy was done by a Shimadzu UV spectrophotometer, Model UV-1800, Japan (Figure 3.24) at 2 nm resolution and 240 nm/min scan speed. This technique is used to quantify the light which gets absorbed and scattered by a sample of a known quantity. It is also known as the extinction and defined as the sum of absorbed and scattered light. Based on this phenomenon presence and concentration of the AgNPs which absorb a specific wavelength of light and possess a unique property of surface plasmon resonance (SPR) was confirmed. The spectrum has also helped in distinguishing AgNPs from others particles present in the solution.

The sample was placed in cuvette between a light source and a photo detector, and the intensity of a beam of light was measured before and after passing through the sample. These measurements were compared at each wavelength to quantify the sample's wavelength dependent extinction spectrum. The absorption spectrum from 190 to 1000 nm was recorded during the test. The data was typically plotted as extinction as a function of wavelength as well as listed in Excel datasheet.

The AgNPs colloidal solutions of minor scale trials (sets – I and II), which have shown better visual colour change pattern on the formation of well stable AgNPs were only considered for UV-Vis spectroscopy. Hence three matters have participated in the colloidal formation, viz; DDW, CP leave extract, and $AgNO_3$, thereby this test was split up into two parts (i.e. Part-I and II). In the first part DDW was taken in reference cuvette and the AgNPs colloidal solution in sample cuvette to get the UV-Visible spectrum. All the AgNPs colloidal considered for the study were tested in the similar fashion. However, spectrums so obtained were having coinciding wavelengths for CP leave extract and $AgNO_3$ combindly present in AgNPs colloidal solution. their traces were segregated in second part of test, conducted by keeping 10% CP leave extract in reference cuvette (i.e. E10S0020 to E10S0100 in Set-I and E10S0001 to E10S0015 in Set-II), only for sample E10S0000 (10% CP leave extract) DDW was kept in reference cuvette, and UV spectrums were obtained. These UV spectrums were helpful in distinguish absorption peak of pure AgNPs from the combined (AgNPs with 10% CP leave extract) wavelength spectrums obtained in the first part.

D) Particle size analysis of AgNPs/CP colloidal solutions

The particle size analyser, Model- Malvern Zetasizer Pro, Version 7.11 (Figure 3.25) was used to analyse the nanoparticles size of the AgNPs/CP colloidal solutions.

The Brownian motion of particles or molecules in suspension causes laser to be scattered at different intensities, like smaller nanoparticles can scatter light more strongly and the intensity goes on reducing with increase in the particle size.

The overall particles size is represented as 'Z average', meaningfully is an intensity weighted mean hydrodynamic size of the ensemble collection of particles measured by DLS. The Z average is derived from a Cumulants analysis of the measured correlation curve, wherein a single particle size is assumed and a single exponential fit is applied to the autocorrelation function. The particle size intensity and its size distribution are analysed using Stokes-Einstein relationship (Equation 3.15) from the analysis of intensity fluctuations of laser [291].

$$\boldsymbol{Dt} = \frac{k_B T}{6\pi\eta R_H} \quad \ldots\textbf{(3.15)}$$

Where;

Dt = Translational diffusion coefficient,
k_B = Boltzmann constant ($1.38064852 \times 10^{-23}$ J/K),
T = Temperature,
η = Absolute viscosity,
R_H = Hydrodynamic radius

The PDI (Polydispersity Index) for DLS typically depicts the intensity of light scattered by various fractions of the particles differing in their sizes and is calculated by $(\text{width/mean})^2$ for each peak. A PDI value of ≤0.1 is considered to be highly mono-disperse, 0.1–0.4 is moderately polydisperse and greater than (>0.4) denotes that the solutions have a highly/broadly polydisperse type of distribution indicating the existence of a large diversity of particle sizes and hence a highly polydisperse solution [246,291].

The sample to be tested was filled in a cuvette of 3 ml size and then test was run (ON). Data and plots for the size distribution profiles of the tested sample particles were acquired using interfaced computer-controlled software developed by Brookhaven Instruments, Holtsville, NY directly. The instrument measures the particles size in terms of particle diameter by using Dynamic Light Scattering (DLS) technique. The measurements were performed with settings; 173° angle and diameter range 0.3 nm to 10 μm, at room temperature (25°C).

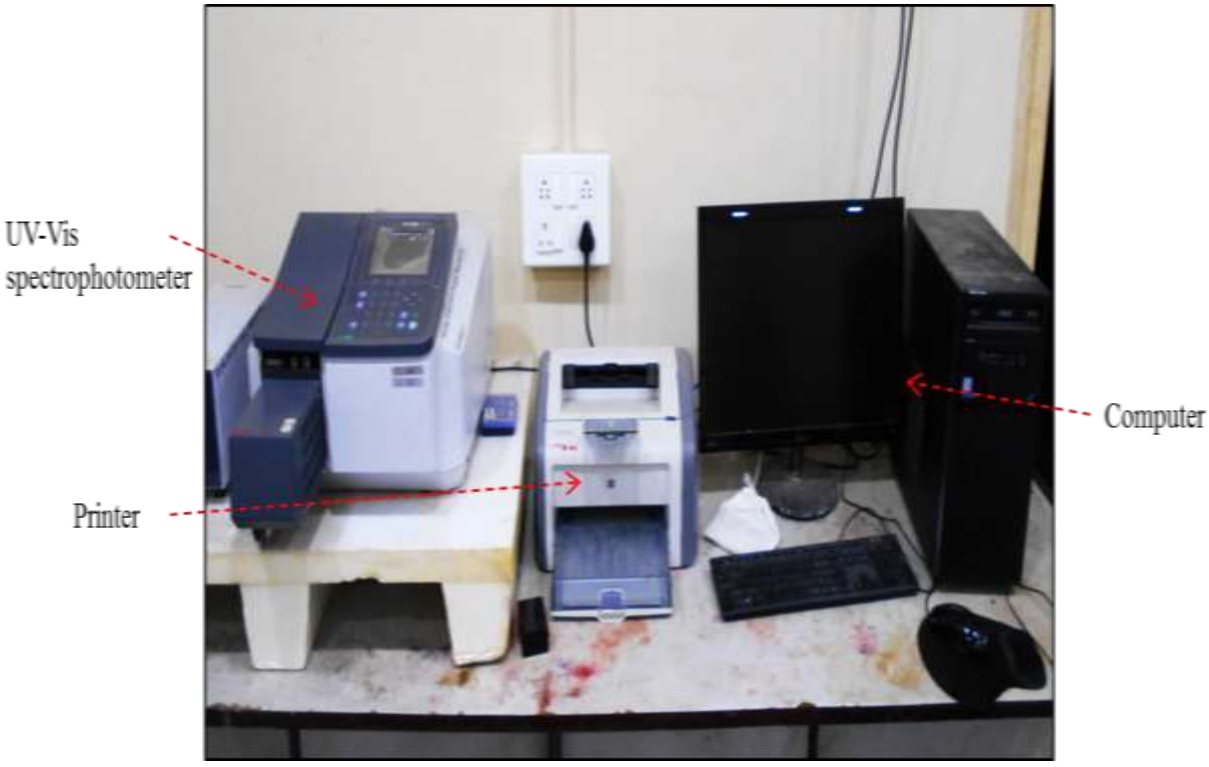


Figure 3.24: Shimadzu UV-Vis spectrophotometer, Model UV-1800

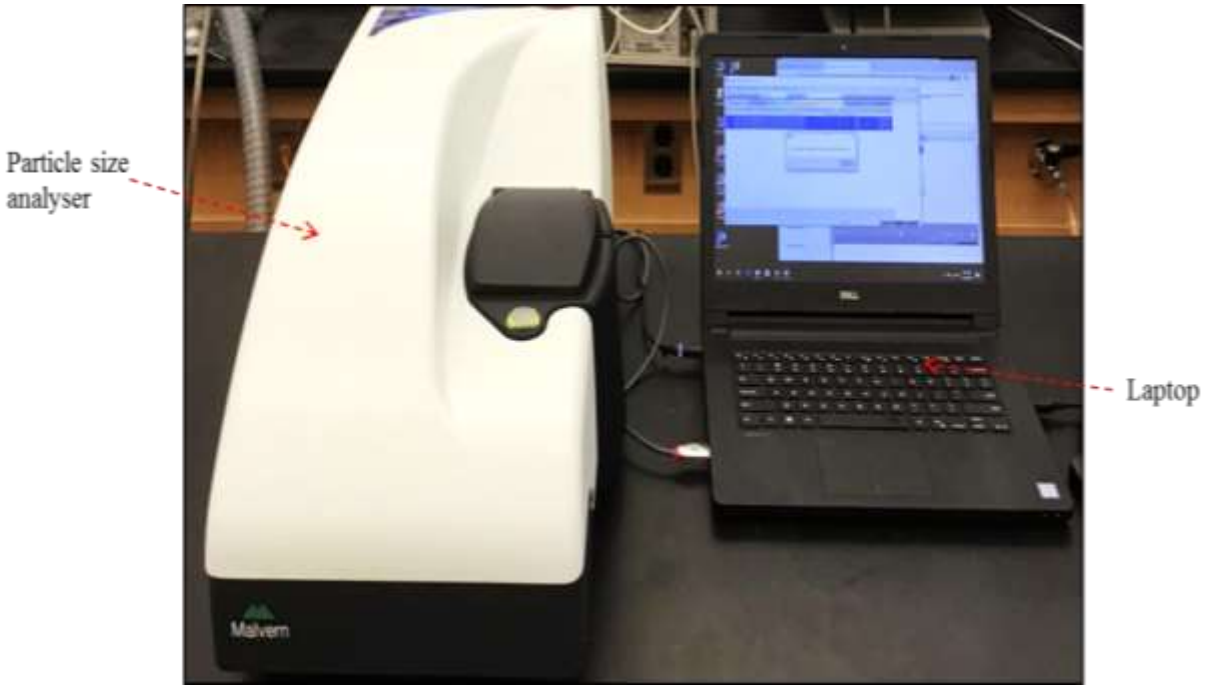


Figure 3.25: Particle size analyser, Model- Malvern Zetasizer Pro, Version 7.11

E) GC-MS analysis of CP leaves extract

The Gas Chromatograph with Mass Sepectrometer (GC-MS), Model- Autosystem XL GC with Turbomass, Perkin Elmer, Germany (Figure 3.26) was used to analyze the mass spectra of the CP leave extract by electron impact ionization (EI) method.

The test was done to identify various chemical compositions of the novel CP leave extract used in the present study. The mass spectra obtained were analysed to define presence, retention time (RT), percent area, molecular weight and chemical formula of various chemical compounds in the substance. These results facilitate in determining scientifically extract contribution as reducing and capping agents during the formation of AgNPs [277], as well as antibacterial agents [292]. The optimised extract (E10) prepared for the AgNPs synthesis, and it was only selected for this assessment.

This analytical method precisely identifies presence of a particular substance in a test sample by making use of combined features of gas-chromatography (GC) and mass spectrometry (MS).

Since both test features are combined, the GC/MS analysis procedure has been described as one unit here. The test included basically three steps;

1) Injection of sample into Gas Chromatograph (GC): The mass range of aqueous extract of the CP leave was checked at a range of 20–620 Daltons (amu). The extract was injected into a port heated up to 250°C to make it volatilized. Helium was used as carrier gas at constant pressure of 100 kPa and flow rate of 20 ml/min.

2) Separation of gaseous components: The volatilized substance flow through the column encapsulated within a special oven with temperature programmed at 70–250°C and increases at the rate of 10°C/min up to injection port's temperature of 250°C. Extract components that are more volatile and smaller in size will travel through the column more quickly than others [232]. This phenomenon facilitated in separation of the various chemical compounds from the extract based on size and/or polarity.

3) Analysis in the Mass Spectrometer (MS): The separated components flow directly out of the column and subjected to mass analysis carried out into the three steps:

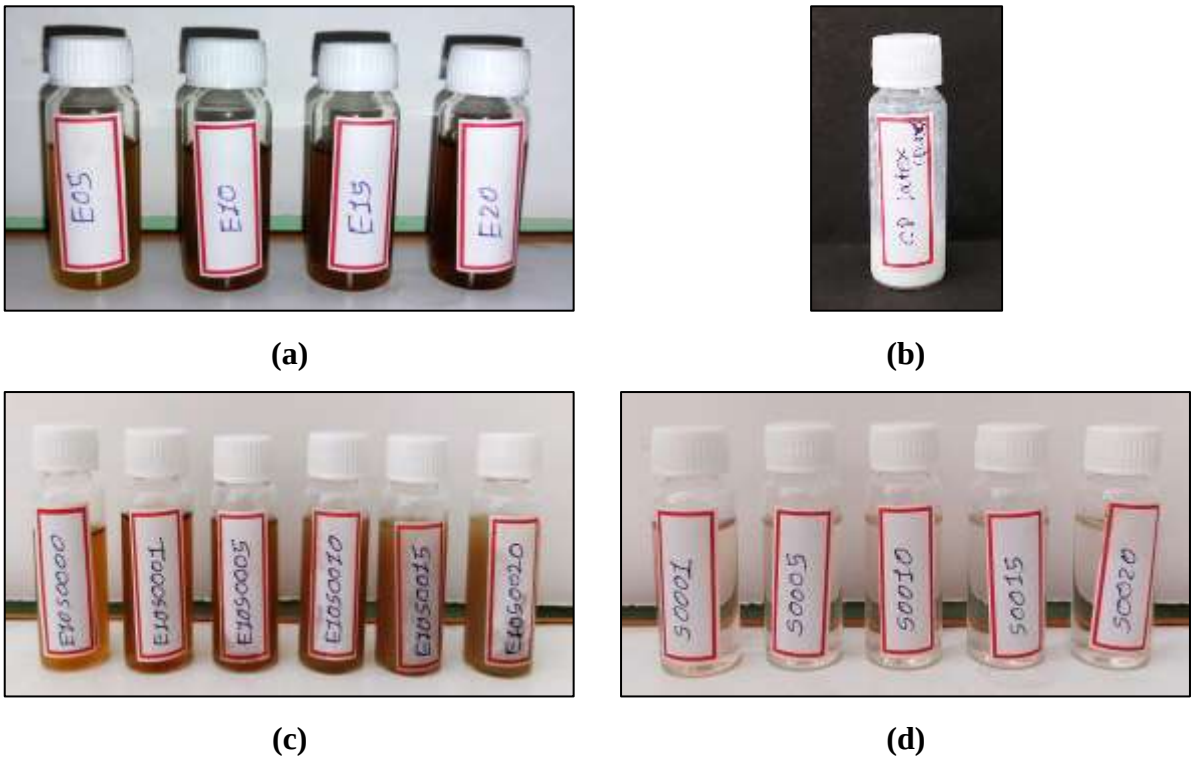
a. Ionization source: The entered gaseous components were blasted with electron ionizer to break them into positively charged ions.

b. Filter: The ions so formed were passed through an electromagnetic field to filter them purely on the basis of their mass. The filter permits only predetermined range of masses to pass through from the ionization source. Thereby, Mass Stability of the extract was checked at constant ±0.1 m/z (mass-to-charge ratio) mass accuracy over 48 hours in the present study.

c. Detector: The numbers of filtered ions were counted by the optical detector and the information sent to an interfaced computer to generate a mass spectrum, i.e. a distribution of ions of different sizes. This mass spectrum was analyzed by using NIST Mass spectra library separation/identification of organic compounds and molecules search programme [293]. This identified the components by comparing each peak with reference libraries; composed of over 275,000 unique spectra (NIST Mass spectra). The compounds within the analyzed sample were quantified by a standard curve of known concentrations of each material, which was auto generated by the computer.

Figure 3.26:

Gas Chromatograph with Mass Sepectrometer, Model- Autosystem XL GC with Turbomass, Perkin Elmer, Germany



(a) **(b)**

(c) **(d)**

Figure 3.27:

MTT assay test samples for Set-I a) CP leave extracts, b) pure CP latex, and Set-II, c) AgNPs/CP colloidal solutions, and d) $AgNO_3$ solutions

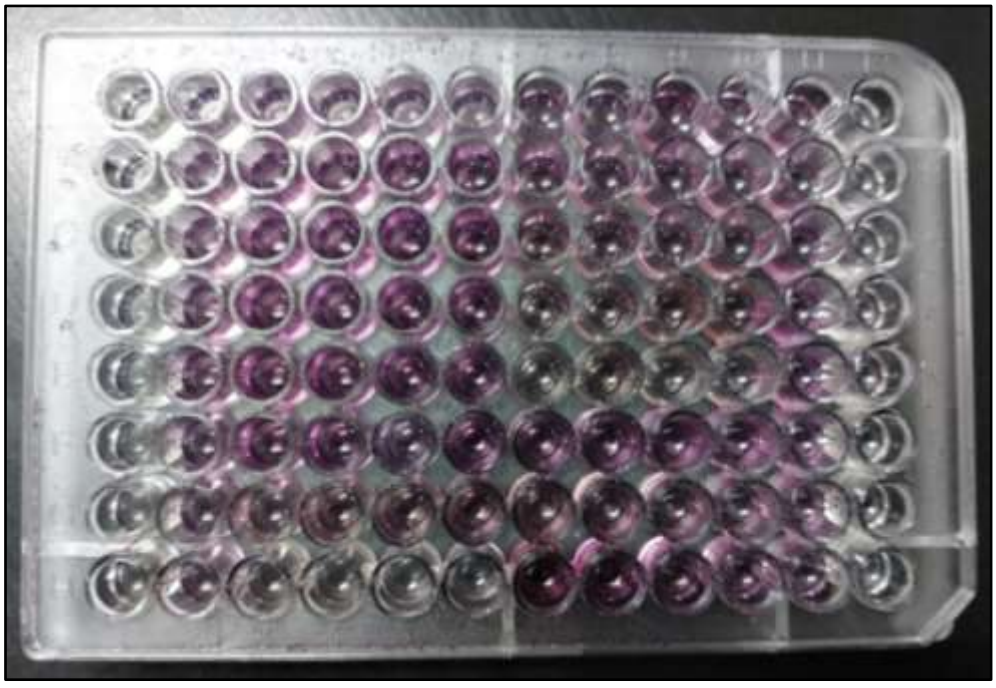


Figure 3.28: 96-well plastic micro plates used for MTT assay

F) MTT assay cytotoxicity test

To investigate the potential toxicity of CP leave extracts and green synthesised AgNPs, two sets of MTT assay cytotoxicity tests [modified from INVITTOX (databank on the use of in vitro techniques in toxicology and toxicity testing) protocol number 112] were performed. The Hep-G2 epithelial cell line of liver derived from Mus musculus (house mouse) was used for the study. This cell line is commonly used in toxicology, cancer research, 3D cell culture, high-throughput screening, and other fields.

In the first set, the toxicity of the CP leave extracts (5%, 10%, 15%, and 20%) [Figure 3.27(a)] were tested against pure CP latex [Figure 3.27 (b)], and in the second set, the toxicity of the AgNPs/CP colloidal solutions [Figure 3.27 (c)] were tested against various concentrations of pure $AgNO_3$ solution (i.e. 1mM, 5mM, 10mM, 15mM, and 20mM) [Figure 3.27 (d)]. To reduce the number of samples, all AgNPs colloidal solutions from set-II and only E10S0020 (which has the lowest concentration in set-I of the minor scale) were chosen for the test. These studies were useful to determine best CP leave extract and the best AgNPs/CP colloidal solution respectively.

The cells were grown as a monolayer at 37°C in a 5% CO_2 atmosphere for 24 hours. The cells were grown in α-MEM (minimum essential medium), which contained a basal medium supplemented with 1% glutamine (used as an auxiliary energy source for rapidly dividing cells as well as a source of nitrogen for protein and nucleic acid synthesis), 10% foetal calf serum (used for growth factors nutrients), and 1% penicillin–streptomycin (used as an antibiotic).

The experiment was carried out in 96-well plastic micro-plates (200-µl capacity) (Figure 3.28) seeded with a cell suspension (5×10^3 cells/well). After 24 hours of growth in the wells, the culture generally become about 60% confluent, indicating 60% cell culture

growth required to achieve an active proliferation state; because only proliferating cells are significant for cell assays. Cells continue to multiply during testing unless the toxicity of the substances being examined start interfering with this process. Thus, the suppression of cell growth (measured as the quantity of total protein in the wells) indicates toxicity of the test specimen [294].

The cells were then exposed to the 5 µl of pure CP latex, various concentration of $AgNO_3$, CP leave extracts, and AgNPs colloidal solutions as mentioned above, and - incubated for another 24 hours. Non-exposed cells with medium were used as a negative control, and all results were compared with these controls. Three concentrations; 0.5 mg/ml, 0.05 mg/ml, and 0.005 mg/ml of 2,4-dinitrophenol were prepared in DMSO (dimethylsulphoxide) and used as a positive control. The medium was removed thereafter and replaced with 100 µl of medium containing MTT reagent or 3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide and incubated for 4 h. After aspirating, the unreduced MTT and 200 µl of DMSO was added to each well to dissolve the MTT formazan crystals.

The cells were then rinsed twice with PBS (phosphate-buffered saline, pH 7.2) buffer. To get preliminary information, light microscopy was used to examine the viability of the cells prior to the addition of sodium phosphate buffer. Following that, 50 µl of sodium phosphate buffer (0.05 mM, pH 8.0) was poured to each well before the plates were frozen for at least 15 min at 70°C. After breaking the frozen cells, the plates were thawed for 15 min, and cell proliferation was measured by assaying the total protein level in the cultures. Sodium phosphate buffer (150 µl) was added to the wells, followed by addition of 50 µl of cold fluorescamine (1.08 mM in acetonitrile). The plates were left at room temperature for 15 minutes before being agitated for 1 minute in a micro-titration plate shaker. The total protein level in each well was determined using a 560 nm wavelength by plate-reading fluorometer.

3.4 PHASE–IV:

DEVELOPMENT OF NANO-COMPOSITES BY TREATING COMMONLY USED BIO-MEDICAL NONWOVEN TEXTILE WITH OPTIMAL AgNPs/CP COLLOIDAL & EVALUATING THEIR PHYSICAL AND FUNCTIONAL CHARACTERISTICS AS PER ASTM STANDARDS

Aim: This phase of study was designed to prepare prototype nano-composites with optimized innovative green-way synthesized AgNPs colloidal solution and evaluating its physical and functional properties.

3.4.1 MATERIALS

i) **Base Material:** The nano-composites were prepared keeping different GSM PP (Polypropylene) non-woven and PV (Polyester-Viscose) non-woven as base material. The materials commonly used in making targeted hygienic bio-medical products; were selected and availed from the respective manufacturer during the study.

Polypropylene (PP) non-woven:

- 30 GSM SMS; widely used in surgical caps, bouffant caps,
- 50 GSM SMS; widely used in medical bed sheets and pillow covers, and
- 45 GSM SMMMS; widely used in surgical apron and drapes.

Polyester-Viscose (PV) non-woven:

Polyester-Viscose (PV, 70:30 blends) nonwoven with 40 GSM was used. This material is used popularly in making distinct medical diapers, surgical aprons/gowns, medical drapes, bandages, gloves, etc.

ii) **CP Extract:** Optimized CP leave extract (E10) viz; B1-E10S00 or F1-E10S0000 (Phase-III, Table 3.7 and Table 3.8 respectively).

iii) **AgNPs/CP Colloidal:** The optimal derived colloidal F4-E10S0010 as per phase III (Table 3.8, Phase-III), synthesized in the laboratory was used.

The reinforced antibacterial efficacy of base nonwoven material was validated for optimal AgNPs/CP loading and pure 10% CP treatment. Thereby in total three types of samples were considered for evaluation in each category of the nonwoven fabrics during the study, viz;

1) ***Reference Sample:*** untreated (Base) PP and PV nonwovens.

2) ***10% concentration CP extract treated samples:*** PP nonwoven -CP and PV-nonwoven -CP.

3) ***Optimal AgNPs/CP treated nano-composite samples:*** PP nonwoven -AgNPs/CP and PV non-woven -AgNPs/CP.

Details of all the developed nano-composite samples along with their preferred application areas are summarized in Table 3.10.

Figure 3.29: Electrical heater [A-ONE Scientific equipments, Volts: 230, Watts: 1500]

Table 3.10: Details of the developed nano-composites

Sr. No.	Material	GSM	Sample Code	Description		Suggested Applications
1.	Polypropylene	30	PP30	Untreated	SMS	Surgical caps, bouffant caps, etc.
			PP30-CP	Only CP treated		
			PP30-AgNPs/CP	AgNPs/CP treated		
2*.	Polyester-Viscose blends	40	PV40	Untreated	(P:V=70:30)	Medical underpads/diapers, surgical aprons/gowns, medical drapes, bandages, gloves, etc.
			PV40-CP	Only CP treated		
			PV-AgNPs/CP	AgNPs/CP treated		
3.	Polypropylene	50	PP50	Untreated	SMS	Medical bed sheets, pillow covers, etc.
			PP50-CP	Only CP treated		
			PP50-AgNPs/CP	AgNPs/CP treated		
4.	Polypropylene	45	PP45	Untreated	SMMMS	Surgical apron and drapes, etc.
			PP45-CP	Only CP treated		
			PP45-AgNPs/CP	AgNPs/CP treated		

** Same material used during Phase-II for Prototype PV-AgNPs/CP nano-composite preparation.*
SMS = Spunbond-Meltblown-Spunbond nonwoven
SMMMS = Spunbond-Meltblown-Meltblown-Meltblown-Spunbond nonwoven

3.4.2 METHODS

3.4.2.1 AgNPs/CP treated samples preparation

The nano-composite textile materials (PP30-AgNPs/CP, PV40-AgNPs/CP, PP50-AgNPs/CP, and PP45-AgNPs/CP) were made in the laboratory. The base PV fabrics were treated with the optimized AgNPs/CP colloidal using the same technique well described in phase -II (Section 3.2.2). Briefly it involved dipping of the known weight (~2 g) of the base fabric into AgNPs/CP colloidal solution by maintaining constant 1:25 bath ratio for 2 hours at room temperature.

However, the method was changed for the PP fabric not absorbing the solution easily owing to its hydrophobic nature. Referring the back track records [299]; the beaker containing PP sample dipped into AgNPs/CP solution was heated to 60±5°C for 20 minutes using electric heater (Figure 3.29). The constant heater temperature was maintained via its inbuilt thermostat control. On heating AgNPs from the solution was gradually absorbed by the PP fibers. All the samples were finally dried by hanging freely for 24 hours in air at room temperature. The dried samples were preserved into a vacuum sealed plastic bag till further analysis to avoid undue interaction effects with air.

3.4.2.2 CP leave extract treated samples preparation

The samples PP30-CP, PV40-CP, PP50-CP, and PP45-CP were made by treating the base nonwoven fabric with the CP leave extract. The same technique of AgNPs/CP nano-composites preparation was followed for the respective base materials.

3.4.3 TEST METHODS

The parent samples (i.e. PP30, PV40, PP50 and PP45), AgNPs/CP nano-composites (i.e. PP30-AgNPs/CP, PV40-AgNPs/CP, PP50-AgNPs/CP, and PP45-AgNPs/CP) and the CP leave extract treated samples (i.e. PP30-CP, PV40-CP, PP50-CP, and PP45-CP) were conditioned at the standard atmospheric conditions for a tropical region; (Temp: $27^{o}C \pm 2^{o}C$ and RH% = 65 ± 2) for 24 hours. The conditioned samples were evaluated for their following application oriented physical, low-stress and comfort-related characteristics mainly.

i) Physical assessment: GSM (gram per square meter), and Thickness
ii) Low-stress properties: Bending modulus, and Crease recovery angle

iii) Comfort-related properties: Air permeability, Moisture management test

The efficacy of nanoparticle in the composite structure is greatly affected by the size and distribution of participating nanoparticle [66]. Hence, an optimal AgNPs/CP colloidal in these regards was used in the nano-composites preparation [Phase-III, Section 3.3.2.2], related tests; VCCO, particle size analysis, UV-VIS spectroscopy, GC-MS analysis and cytotoxicity test [Phase-III, Section 3.3.2.2.1], were not replicated.

Whereas, functional properties of the nano-composite can be verified via the tests like, antibacterial activity, SEM, FTIR, and UV-Transmission [34,41]. The tests were performed for all the PP nonwoven -AgNPs/CP composites using standard procedures [phase-II, Section 3.2.3). Since these functional characteristics of PV-AgNPs/CP composite have already been evaluated exhaustively in Phase-II, the test values are roll over to this phase for the comparative analysis.