3

Materials and Methods

3.1 General

The details of chemicals, solvents, and instruments used in this study are discussed in 3.1.1 and 3.1.2.

3.1.1 Chemicals and solvents

Distilled water was collected using Zeneer Power Water Purification System (ZE1100604), and it used throughout the study. Analytical (AR) and Laboratory reagent (LR) grade synthetic compounds and solvents were used. Gold chloride, Muller Hinton Agar, and Chitosan were purchased from HIMEDIA. Graphite powder, Cetyl alcohol was purchased from Loba Chemie. Gelatin, Sodium alginate were purchased from the Sulab laboratory. Glycerol was purchased from Hipure. NaOH and Urea were purchased from EMPLURA and Reachem Laboratory respectively.

3.1.2 Instruments

3.1.2.1 Instruments used for sample preparation

All the chemicals and prepared samples were weighed using an electronic balance (Shimadzu (AUW220D), Model No:D445910303 and DA50000702). Digital Ultrasonic cleaner (LMUC series-CL 2259,100W) was utilized to prepare nanoparticles and disperse the prepared samples. Sigma heating mantle (SL.NO:213407) and Water bath (Heating mantle, SL.NO:763) were used for extraction purposes. Ultrasonic homogenizer was used in the preparation of nanocomposites and dispersing the samples (Biologics, Inc. Model 300 V/T). Microwave oven (LG ECN:MS1947C/01) and Hot air Oven (Sigma Scientific, 181818 sizes, 1750W, SL.NO:21470) was used in the synthesis and applications part. pHep pH tester (HI98107) was used in pH analysis. The selected samples were stirred and heated using a Magnetic stirrer (REMI 1 MLH LGMS-6073). Samples were stored in a refrigerator (REMI-RLR-300).

3.1.2.2 Instruments used for characterization and biological studies

All the prepared samples in this study were characterized using the following instruments.

- UV Spectrometer (Biospec-nano (230V)

- FTIR (MIRacle 10 Shimadzu)
- 3D Optical Profilometer (Zeta -20)
- X-ray Diffractometer (X'pert powder PAN analytical)
- Field Emission Scanning Electron Microscope (MIRA3 FESEM, TESCAN)
- EDS with SUTW-SAPPHIRE model detector.
- Raman spectrometer (R-3000 QE spectrometer)
- Thermogravimetric analyzer (EXSTAR-SII-6300)
- Zwick / Roell Z010 Tensile meter

The following instruments are used in biological activities.

- UV-Chamber
- Incubator
- Esico µP Photo colorimeter (Model 1311)
- ELISA multiwell plate reader (Thermo Multiskan EX, USA)
- Fluorescence microscope (Nikon Eclipse. Inc. Japan)

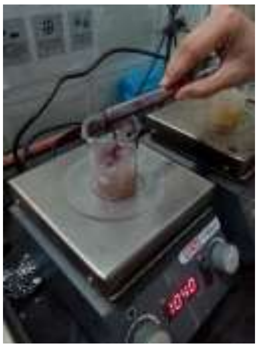
Photographs taken during sample preparation

Distillation of solvents

rGO preparation under refluxing method

Homogenization of nano composite

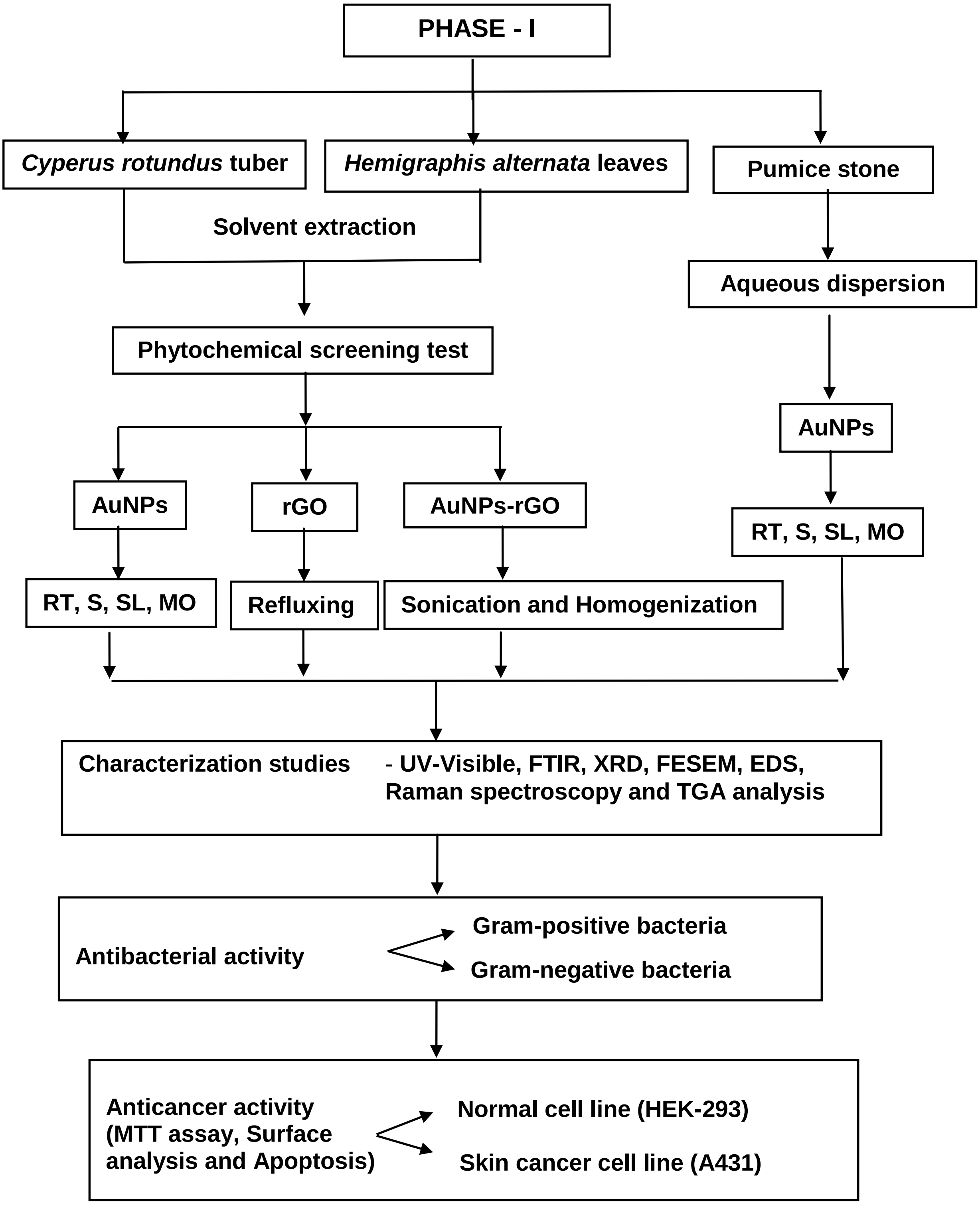
Cream preparation



Addition of AuNPs into blank

Preparation of skin substitutes

The present study divided into three phases. Phase I are represented in the following flow chart.



3.1.3 Preparation of stock solutions

Hydrogen peroxide (30%) was prepared using 30 mL H_2O_2 in 100 mL distilled water, and it was stored in a brown bottle to avoid oxidation of H_2O_2. Hydrochloric acid (10%) solution was prepared by dissolving 10 mL concentrated hydrochloric acid in 100 mL distilled water. Dissolving Gold chloride 0.1017g in 100 mL provided an aqueous solution of gold chloride (3 mM) and it refrigerated for further use. Glacial acidic acid (2 mL) was sonicated with 98 mL distilled water in a standard measuring flask for 5 min. This solution was used as a solvent for chitosan.

3.2 Samples used in this study

Three samples (**Figure 1a-c**) were used in this study based on their traditional knowledge. The details of the selected samples are given in **Table 6**.

Table 6. The profile of *Cyperus rotundus*, *Hemigraphis alternata*, and Pumice stone

S. No	Sample details	Samples used in this study		
		Cyperus rotundus	***Hemigraphis alternata***	**Pumice stone**
1	Acronyms	CR	HA	PS
2	Kingdom	Plantae	Plantae	-
3	Family	Cyperaceae	Acanthaceae	-
4	Common name	Korai kilangu	Murikootti	-
5	Parts used	Tuber	Leaves	Whole stone
6	Colour	Brown	Greenish violet	White

Figure 1a. CR

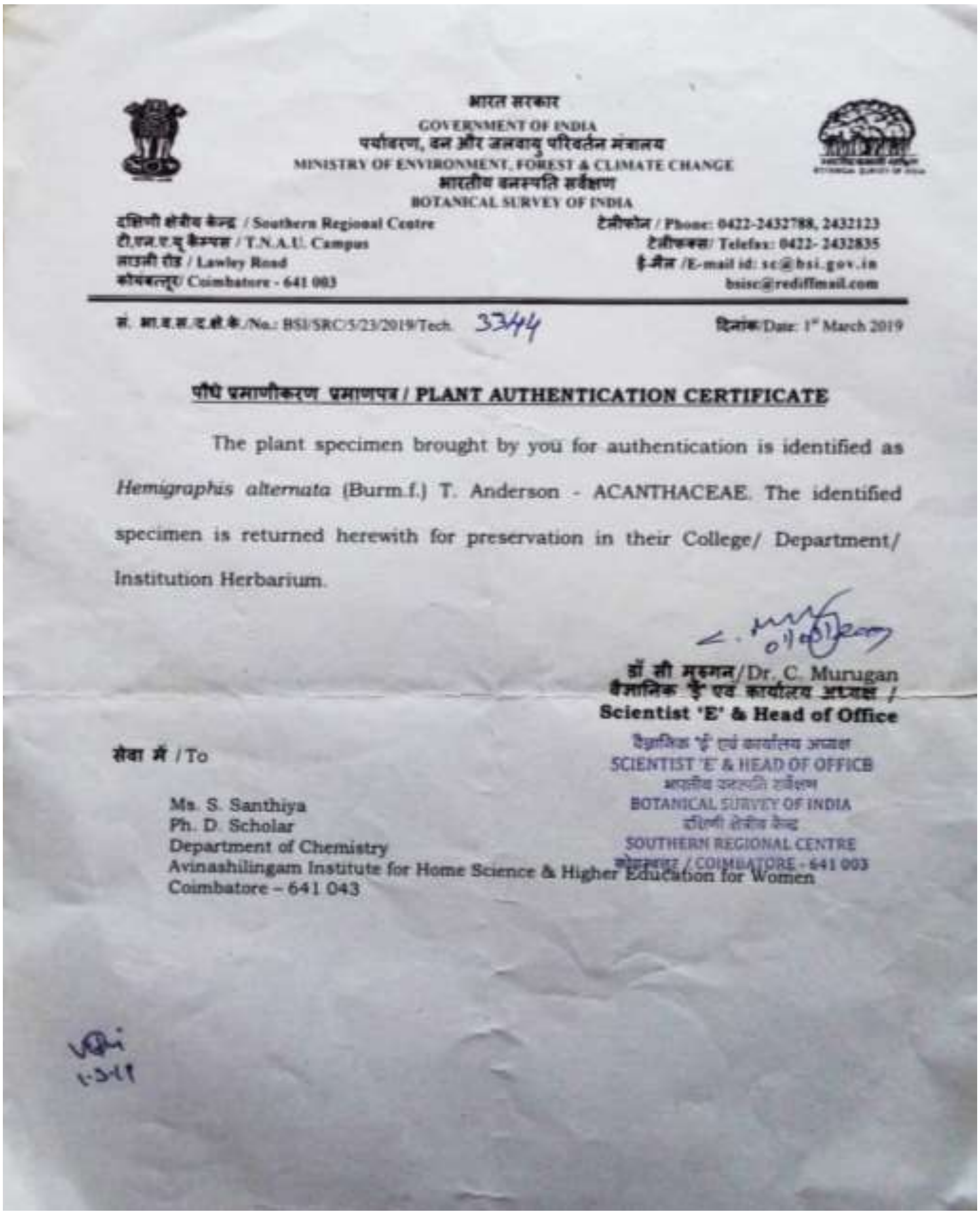
Figure 1b. HA

Figure 1c. PS

Figure 1a-c. Photographs of selected samples a) *Cyperus rotundus* tuber, b) *Hemigraphis alternata* (Burm.f.) leaves and c) Pumice stone

3.2.1 Collection and identification of plant sources (CR and HA)

Cyperus rotundus tuber (CR) and pumice stone (PS) were purchased from a local shop in Coimbatore, Tamilnadu, India. The leaves of *Hemigraphis alternata* (Burm.f.) T. Anderson (HA) was collected from AIHSHEW, Coimbatore in the month of January. The authentication number for this plant is BSI/SRC/5/23/2019/Tech 3344, and this is given by the Botanical Survey of India (**Figure 2**), TNAU, Coimbatore -43.



भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
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सं. भा.व.स.द.क्षे.के./No.: BSI/SRC/5/23/2019/Tech. 3344

दिनांक/Date: 1st March 2019

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Hemigraphis alternata* (Burm.f.) T. Anderson - ACANTHACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

01/03/2019

डॉ सी मुरुगन/Dr. C. Murugan
वैज्ञानिक 'ई' एवं कार्यालय अध्यक्ष /
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कोयंबत्तूर / COIMBATORE - 641 003

सेवा में / To

Ms. S. Santhiya
Ph. D. Scholar
Department of Chemistry
Avinashilingam Institute for Home Science & Higher Education for Women
Coimbatore – 641 043

1-3-19

Figure 2. Scanned copy of plant authentication certificate of *Hemigraphis alternata* (Burm.f.)

The leaves of *Hemigraphis alternata* initially washed with running water to remove the external impurities. The water droplets on the leaves were removed using absorbing paper then dried at room temperature for one week. The dried leaves were ground sparingly and stored in a plastic container.

3.2.2 Dispersion of pumice stone

The pumice stone was rubbed and ground with mortar and pestle. The fine powder of pumice stone was sieved using a cotton cloth and stored tightly in screw cap bottles. Fine powder of PS was extracted by sonication and homogenization for one hour. Distilled water was used for this study as a solvent. The mixture was filtered and the white turbid filtrate used for further analysis.

3.2.3 Sequential solvent extraction of CR and HA-refluxing method

The dried tuber portion of *Cyperus rotundus* was ground sparingly and stored in a plastic container. Extraction of CR (15g) and HA (10g) was carried out under refluxing method using various solvents such as Petroleum Ether (PE), Ethyl Acetate (EA), Chloroform (CF), Acetone (A), Carbinol (C), Ethanol (E) and Water (W). Refluxed solvent extracts were filtered using the cotton plug and the solvents were removed. The weight of the moisture-free solvent extracts was taken and stored in a glass container.

3.3 Phytochemical assessment of solvent extracts of CR and HA

Phytochemical assessment of different solvent extracts of CR and HA has been carried out based on **Thamaraiselvi *et al*., 2012; Vimalkumar *et al*., 2014; Vijisaral and Arumugam, 2014**. This assessment helps to discover the materials to be involved in nanoparticle synthesis and biological activity studies. The procedure of phytochemical screening of these plants are given in **Table 7**.

Table 7. Phytochemical assessment of selected plant extracts

S. No	Test	Reaction mixture	Observation
1	**Alkaloids**		
1 a	Dragendorff	Extract + Dragendorff reagent	Red precipitate
2	**Flavonoids**		
2 a	NaOH	Extract + NaOH + HCl	Yellow-orange
2 b	H_2SO_4	Extract + Concentrated H_2SO_4	Orange colour
2 c	NH_4	Extract + Aqueous NH_4 + Concentrated H_2SO_4	Yellow-orange
2 d	Shinoda's	Extract + Ethanol + Magnesium + Concentrated HCl	Pink or magenta
2 e	$ZnCl_2$	Extract + Zinc dust + Concentrated HCl	Magenta colour
2 f	$FeCl_3$	Extract + Neutral $FeCl_3$	Blackish red
2 g	Lead acetate	Extract + Lead acetate	Orange to crimson
3	**Sterols**		
3 a	Liebermann-Burchard	Extract + Chloroform + Acetic anhydride + Drops of H_2SO_4	Dark red colour
3 b	Salkowski's	Extract + Chloroform + Concentrated H_2SO_4	Blue layer
4	**Terpenoids**		
4 a	Liebermann-Burchard	Extract + Chloroform + Acetic anhydride + Drops of H_2SO_4	Dark green colour
5	**Anthraquinones**	Extract + Chloroform + NH_3 (10%)	Pink layer
6	**Anthocyanins**		
6 a	NaOH	Extract + 2M NaOH	Blue-green colour
7	**Proteins**		
7 a	Ninhydrin	Extract + Ninhydrin (aqueous)	Purple colour
7 b	Biuret	Extract + $CuSO_4$ (2%) + Ethanol (95%) + KOH	Pink ethanolic layer
8	**Phenols**		
8 a	Ferric chloride	Extract + Ferric chloride (5%)	Deep blue
8 b	Lead acetate	Extract + Lead acetate	Yellow precipitate
9	**Quinones**	Extract + Concentrated HCl	Yellow precipitate
10	**Carbohydrates**		
10 a	Molisch's	Extract + Molisch's reagent + Concentrated H_2SO_4 + H_2O	Red colour
10 b	Fehling's	Extract + Fehling's (A and B) solution	Red precipitate
11	**Saponins**		
11 a	Froth test	Extract + H_2O	Formation of form
11 b	Foam test	Extract + H_2O	Formation of form

3.4 Synthesis of gold nanoparticles using CR, HA and PS

3.4.1 Preparation of aqueous extracts of CR, HA and PS for nanoparticle synthesis

Aqueous extracts of HA (1g), CR (1g), and the fine powder of PS (3g) were sonicated for 15 min with 100 mL distilled water separately, and then it was filtered using a cotton plug. The aqueous extract of CR and HA represented as CRa and HAa respectively. The CRa, HAa, and PS in water are represented as CRablank, HAablank, and PSablank, respectively. The filtrate of all samples was stored in the refrigerator at 6°C, and it was taken for gold nanoparticles synthesis.

3.4.2 Synthesis of gold nanoparticles using CRablank, HAablank and PSablank

The gold nanoparticles were synthesized using CRablank, HAablank and PSablank by conventional methods such as Microwave heating (MO), Sonication (S) Sunlight irradiation (SL) and Room temperature (RT). The various ratios of samples and auric chloride were treated to form gold nanoparticles in a short time. The colour changes of nano solution were noted visually.

3.4.2.1 Room temperature method

The gold nanoparticles were synthesized using CRablank, HAablank and PSablank (in various ratios) and equal ratios of $HAuCl_4$ (1:1, 2:1, 3:1, 4:1, and 5:1). The solution was kept at atmospheric temperature (28-35°C) without any disturbance. The colour change of greyish pink, violet and bluish grey was obtained using CRablank, HAablank and PSablank respectively. These visual colour changes confirm the reduction of auric chloride.

3.4.2.2 Sonication method

Greyish pink, Violet, and Bluish grey colour gold nanoparticles were synthesized using CRablank, HAablank and PSablank respectively. Various ratios of samples and equal ratios of gold chloride (1:1, 2:1, 3:1, 4:1 and 5:1) were treated under the sonication method.

3.4.2.3 Sunlight irradiation method

CRablank, HAablank and PSablank were used to synthesize greyish pink, violet and pink colour gold nanoparticles. The test samples were kept in an un-disturbing place under sunlight (30-37°C) and the mixture was obtained by treating equal ratios of gold chloride and different ratios of samples (1:1, 1:2, 1:3, 1:4 and 1:5).

3.4.2.4 Microwave heating method

Different ratios of CRablank, HAablank and PSablank with an equal ratio of gold chloride (1:1, 2:1, 3:1, 4:1 and 5:1) were micro-waved using a conventional microwave oven (800W). During the formation of the nanogold, the colour of the mixture turned greyish pink using CRablank and violet colour using HAablank and PSablank.

3.4.3 Concentration variation study of gold nanoparticles synthesis

The concentration variation study was carried out to fix the suitable ratios for the formation of nanoparticles in a short time. The following three methods (Room temperature, Sunlight irradiation and Microwave heating) were employed in the concentration variation study based on section 3.4.2.1-3.4.2.4.

3.4.3.1 Room temperature method

Compared to all the methods, HAablank showed a minimum time difference to form gold nanoparticles. Hence, the room temperature method was used for HAablank to synthesise gold nano because it is a very simple and conventional method. HAablank was treated in different ratios, with gold chloride (in equal ratio) (1:1, 2:1, 3:1, 4:1 and 5:1) and the formation of violet colour gold nano was noted. Different ratios (1,2,3,4 and 5) of gold chloride and equal ratio of HAablank (1:1, 2:1, 3:1, 4:1 and 5:1) were treated. Colour change of violet and ash blue colour from yellowish-brown colour indicates the reduced gold ions by HAablank.

3.4.3.2 Sunlight irradiation method

PSablank (different ratios) and gold chloride (equal ratio) were introduced to sunlight at 30-37°C. The formation of the pink colour was observed in the mixture. Likewise, the treatment of gold chloride with PSablank (1:1, 2:1, 3:1, 4:1 and 5:1) reduced the gold into gold nanoparticles and which is confirmed by the colour change from pale yellow to bluish grey.

3.4.3.3 Microwave heating method

Different ratios of CRablank mixed with equal ratio $HAuCl_4$ (1:1, 2:1, 3:1, 4:1 and 5:1) and it was heated in a microwave oven. The colour changes from yellowish brown to greyish pink indicates the reduction of gold ions.

3.5 Preparation of reduced graphene oxide (rGO) using CRablank and HAablank

CRablank (400 mg) and HAablank (250 mg) were sonicated for 15 minutes at 40°C with 30 and 60 mL distilled water respectively. The sonicated solutions were filtered using a cotton plug, and it was used for rGO preparation.

3.5.1 Preparation of graphene oxide (GO) (Hummers and Richard, 1958; Firdhouse and Lalitha, 2014)

Graphite powder (10g) was treated with concentrated H_2SO_4 (120 mL). This reaction mixture was stirred one hour constantly in an ice bath. $KMnO_4$ (20g) was added gradually over a period of one min at temperature 20°C for 3 hours. To this mixture, distilled water was added, and the temperature was maintained at 100°C. Finally, 30% of H_2O_2 was added until the mixture turned yellow colour. The obtained mixture was filtered using 10% HCl to remove the metal ions and repeated washing with distilled water to obtain a powder of pure graphene oxide (GO).

3.5.2 Preparation of reduced graphene oxide (rGO)

The prepared CRablank (30 mL), HAablank (60 mL) and the prepared GO (30 mg) were sonicated in 30 mL distilled water for 15 min separately. The sonicated GO was added to each solution. The mixture was homogenized for 10 min by the 30 pulse range. Then the solution was refluxed for 2h for CRablank and 10h for HAablank until the mixture colour changed into the black from brown. The reduced mixture was again sonicated for one hour, and it was further homogenized for one hour to obtained fine dispersed particles. All final solution was centrifuged. The filtrate and residue were taken for further analysis. The prepared reduced graphene oxide using CRablank and HAablank were represented as CRarGO and HAarGO, respectively.

3.6 Preparation of AuNPs-rGO composite (Homogenization method)

CRablank and HAablank aided gold nanoparticles (CRaNP and HAaNP), and reduced graphene oxides (CRarGO and HAarGO) were taken in the ratio of 1:1. The prepared samples were dispersed in distilled water (10 mL) for one hour under sonication, followed by homogenization (1h) to form a nanocomposite. The formed nanocomposite using CRablank and HAablank were designated as CRaNC and HAaNC, respectively. The colour of the mixture turned into blackish pink and blackish violet for CRaNC and HAaNC respectively.

3.7 Characterization of synthesized metal and non-metallic nanoparticles

Synthesized AuNPs, rGO and AuNPs-rGO composite was characterized to analyze their formation, functional groups, shapes, size, distribution and thermal stability.

3.7.1 UV-Visible Spectroscopy

The bio-reduction of Au ions to Au nanoparticles was confirmed using UV analysis. The formation of synthesized AuNPs, rGO and AuNPs-rGO composite using CRablank, HAablank and PSablank were monitored in UV-Visible Spectrometer. The samples were analyzed in the range of 220-800 nm. First, a drop of solution was placed in a sample path of the instrument, and then the Surface Plasma Resonance (SPR) band of the samples was recorded.

3.7.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR (MIRacle 10 Shimadzu) was utilized in this analysis (between 4000-400 cm^{-1}) and the important bioactive functionalized groups in the prepared samples responsible for the reduction of nanoparticles were identified.

3.7.3 X-Ray Diffraction Spectroscopy (XRD)

The crystalline size of gold nano is calculated by measuring the full width of the half peak maxima at 2θ pattern where the radiation is located **(Monshi *et al.*, 2012)**. The Scherrer equation ($D= k\ \lambda/\beta \cos \theta$) was used to find the crystalline size of the prepared gold nanoparticles. Here, a wavelength of X-ray (A˚)-λ; crystallite shape factor-k; diffraction broadening – β **(Alexander and Harold, 1950)**. XPERT-PRO instrument was used in XRD analysis with CuKα radiation at 30 mA and 45 kV. Samples were prepared for XRD by placing the prepared nanoparticles onto a small transparent glass plate (2x2 cm) and dried under vacuum desiccator.

3.7.4 Thermo Gravimetric Analysis (TGA/DTG)

Thermogravimetric analysis was conducted under a nitrogen atmosphere using a thermogravimetric analyzer (EXSTAR-SII-6300). The sample decomposition between 30°C to 1200°C was analyzed, and combined TGA/DTG helps indicate the sample's sharp decomposition point.

3.7.5 Raman Spectroscopy

Raman spectrometer (R-3000 QE) provided information about the reduced graphene oxide formation within the 500 to 2000 cm^{-1} analytical range.

3.7.6 Field Emission Scanning Electron Microscope (FESEM)

FESEM (MIRA3, TESCAN) helps to analyzes the surface texture and the size of synthesized AuNPs, rGO and AuNPs-rGO composite. The prepared nanoparticles were coated onto a glass plate (8 mm) and allowed in vacuum drying. A small piece of double-sided adhesive carbon tape is placed on an aluminium stub, and the glass is placed on the upper portion of carbon tape. To make the glass plate conductive, a small strip of carbon tape is placed between the glass plate and aluminium stub. This technique is helpful to avoid the distortion of the quality of the sample image. The samples were sputtered at 10 mV for 60 seconds using the SC7620 Sputter coater under the nitrogen atmosphere. Samples were taken in a sample holder and analyzed in FESEM with different magnification up to 30 kx, working distance (15 to 4 mm), high voltage (10 kV) and view field (µm to nm).

3.7.7 Energy Dispersive Spectroscopy

The elemental analysis of the prepared samples was performed by using Energy Dispersive Spectroscopy with SUTW- SAPPHIRE type detector. Apex software was used for examining the samples. The elements in different shells (K, L, M, *etc*.) also identified. The distribution of the elements was present in the samples were identified by mapping.

3.8 Biological activities of prepared nanoparticles

Prepared nanoparticles were tested for their antibacterial and anticancer activity which helps to screen the samples for further applications.

3.8.1 Antibacterial activity of prepared samples

The antibacterial behaviour of selected samples and their nanoparticles was performed using the method of Agar-well diffusion. Four human pathogens such as gram-negative (*Escherichia coli* and *Salmonella paratyphi*) and gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) are were used in antibacterial activity.

3.8.1.1 Sample preparation

CRaNP, CRarGO, CRaNC, CRablank, HAaNP, HAarGO, HAaNC, HAablank, PSaNP, and PSablank were prepared as mentioned in 3.4.3, 3.5 and 3.6. All the samples were dried at room temperature and weighed. Then the samples were sonicated with distilled water for half an hour and homogenized for 1h. The complete dispersed solution was used for further study.

3.8.1.2 Evaluation of antibacterial activity using Agar-well diffusion method

Initially, all apparatus was cleaned with soap solution and water. Then removed moisture for 1h in a hot air oven. The dried petri dishes were again cleaned with alcohol, and it was UV sterilized for half an hour before using. The Agar solution was prepared by treating distilled water (300 mL) with Muller Hinton Agar and Agar type II (3:1). Sonication and microwave heating were applied to the mixture for 15 and 10 min respectively. The prepared hot Agar mixture was added into sterilized Petri dishes, and it was kept for some time to solidify. After that well (0.5 cm) was created, and bacterial strains (10 µL) was swabbed in zig-zag motion on the petri dishes. Various concentrations (150, 200 and 250 µL) of each sample (40 µL) were added, and it was kept in an incubator for 24h at 37ºC. The bacterial inhibition zone was measured after the inhibition period was over (24h). Ciprofloxacin which is commercially available standard was used to compare the results.

3.8.2 Anticancer activity of prepared samples

In this study, the toxicity or cell viability of prepared samples on human normal cell lines (HEK- 293) and skin cancer cell lines (A431) was evaluated by MTT assay, surface morphology and dual staining analysis **(Santhiya and Lalitha, 2020a)**. The detailed procedure is given in **Appendix I**.

3.8.2.1 Collection and maintenance of cell lines

The Human skin cancer cells (A431) and Human embryonic kidney cells (HEK-293) were purchased from NCCS, Pune, India. The A431 and HEK-293 cell lines were properly maintained with providing essential supplementation in DME medium under CO_2 atmosphere at 37°C.

3.8.2.2 Evaluation of cell viability using MTT assay

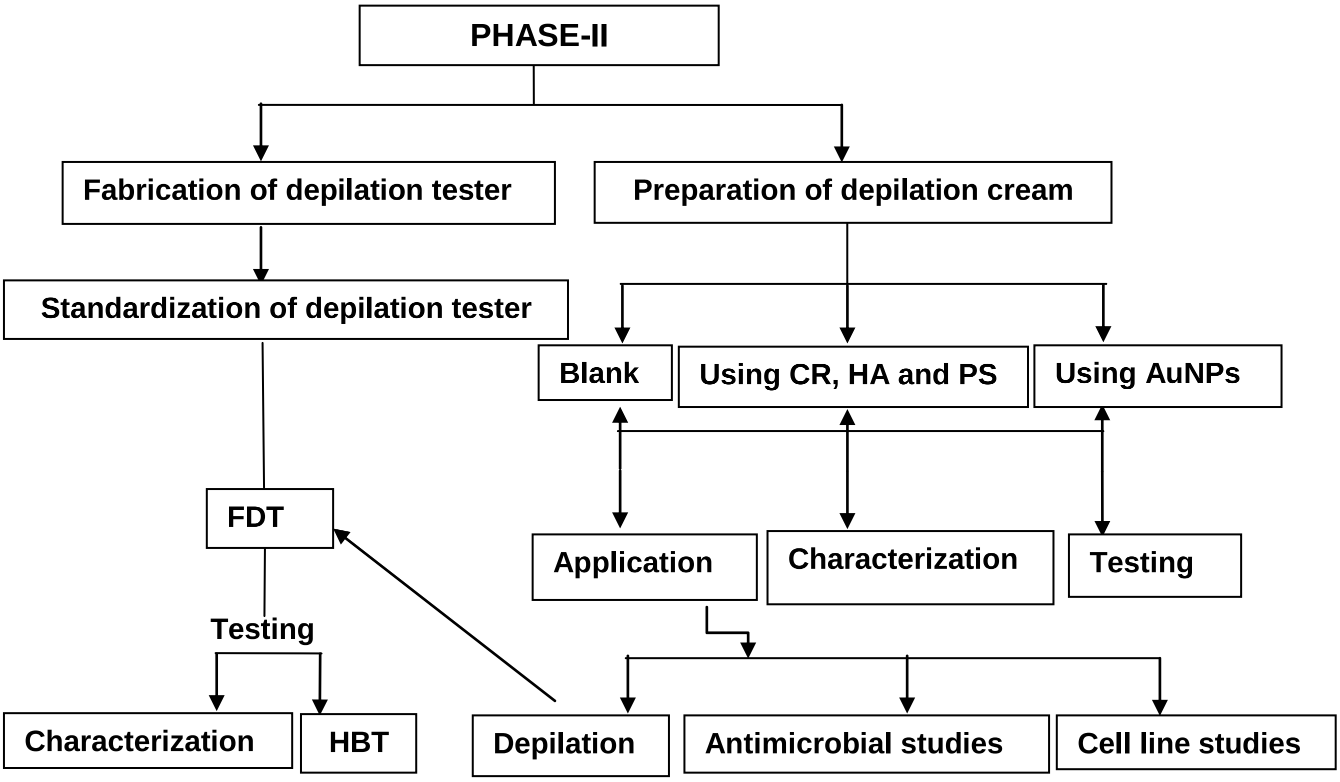
The IC_{50} value of prepared samples were taken using the MTT assay. A431 and HEK-293 cells (1×10^5 cells/well) were cultured in a 96-well plate. The culture medium was removed, and then prepared samples were added in it. The cell culture was incubated for two days. MTT (100 µL) assay was added in each sample containing wells. Then it was further incubated for four hours at 37ºC. The supernatant solution was replaced by DMSO (50 µL) and allowed to 10 minutes incubation. The OD value (ELISA multi-well plate reader) was measured (at 620 nm) after the formazan crystals are solubilized.

$$\text{Cell viability (\%)} = \frac{\text{OD value of experimental sample}}{\text{OD value of experimental control}} \times 100 \quad \text{............ Equation 1}$$

3.8.2.3 Apoptotic inhibition (Dual staining method)

For PI staining, the propidium iodide (100 mg/mL) and for Acridine Orange/Ethidium Bromide Staining, AO (100 mg/mL)/EtBr (100 mg/mL) was dissolved in distilled water. This mixture is used as a dye solution for this study. The cancer cells (1×10^5 cells/mL) were treated with prepared dye mixture in microscope coverslips and incubated for two minutes. The cancer cell lines washed with PBS (pH 7.2) before and after staining. The changes of cell structure were observed in a fluorescence microscope at 520 nm (for AO/EtBr staining) and 480 nm (for PI staining).

The overview of phase II are given in the following flow chart.



3.9 Standardization of *in vitro* method to evaluate depilation time

Commercially available depilatory creams and prepared depilatory samples were tested for their depilation efficacy using a simple depilation tester. This method was standardized using a simple glass apparatus. The details of fabrication are given in 3.9.1.

3.9.1 Fabrication and standardization of *in vitro* glass depilation tester using commercial depilatory creams

Based on **Harvey and Roberta (1968)** a simple *in vitro* glass depilation tester was standardized to evaluate the depilatory action of the prepared creams by calculating the breaking time of hair. Std (V) and Std (F) commercially available depilatory creams were used for the standardization. Both the creams were taken in 5 different concentrations (1, 2, 3, 4 and 5 mg) and coated on a different area of the hair (1, 2 and 3 cm). The total length of the hair was 10 cm, and the average thickness was 0.02 mm±0.009. The time of the hair broken was noted using a stopwatch. Triplicate results were carried out. Mean, and Standard Deviation (SD) was calculated for the obtained depilation results using Microsoft Excel 2007. The different parts of the fabricated glass depilation tester are given in 3.9.1.1 and **Figure 3**.

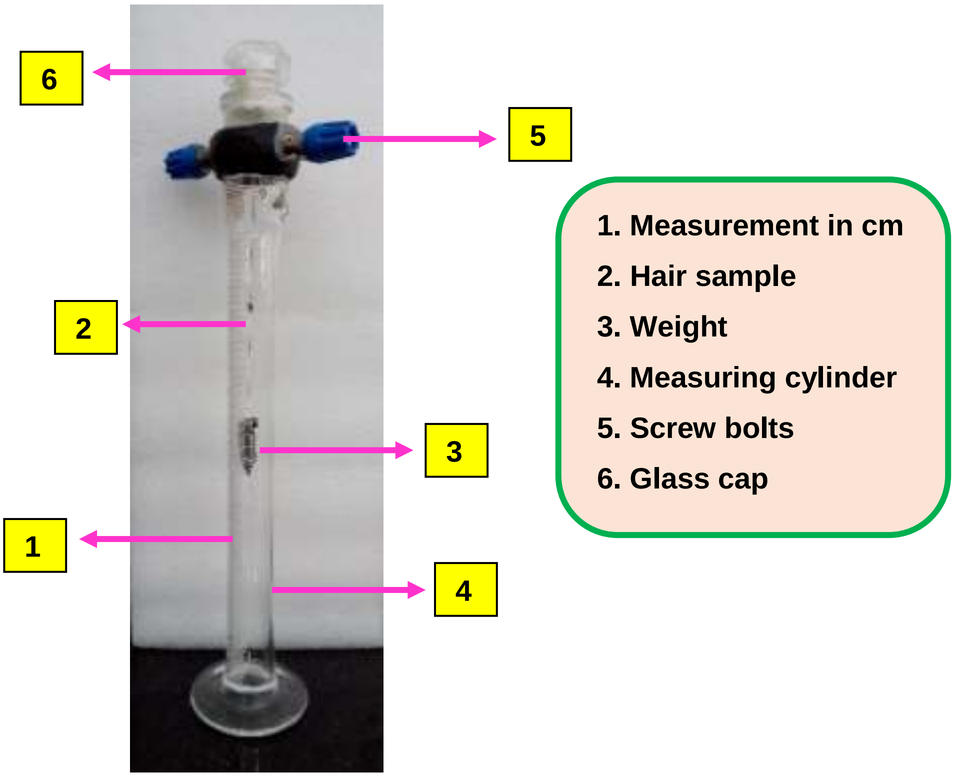


Figure 3. Photographic representation of fabricated depilation tester

3.9.1.1 Different parts of fabricated depilation tester (FDT)

1. Measurement (in cm)

Measurements in FDT was used to identify the total length of the hair, cream coated area on hair, and measure the hair elongation.

2. Hair sample

In this study, human hair was used as a sample. That is arrested by adjustable screws (on top) and weight (on bottom).

3. Weight

Weight (5g) used in FDT is made of aluminium which used to break the weakened hair samples by elongation. It contains sharp edges to maintains the gravity.

4. Measuring cylinder

All the depilation study was carried out within the cylinder. This was completely made of glass.

5. Screw bolt

This screw bolt was used in fabricated glassware. Two adjustable aluminium screws were used to arrest the hair samples from fall down.

6. Glass cap

Moisture and atmospheric air may be affecting the efficiency of the creams. Hence, the glass cap on the top of the FDT was used to reduces the interruption of air and moisture.

3.10 Formulation of depilatory creams using selected samples

3.10.1 Selection of chemicals and samples

Based on literature, the samples used in this study were chosen. Cetyl alcohol ($C_{16}H_{34}O$) was used as thickener and emulsifying agent **(Mohiuddin, 2019)** and it enhances the texture and spread-ability of the cream. Fuller's Earth (Multani mitti) is a naturally available clay which is used as a cleaning agent **(Bhutkar and Shah, 2019)**, anti-ageing agent, absorbent **(Rashmi *et al*., 2017)** and cosmetic-related applications. NaOH was used as a pH adjuster **(Park *et al*., 2019)** and keratin degradation agent **(Horvath, 2009; Valeika *et al*., 2009)**. Urea was used as an accelerator **(Atkin *et al*., 2015)** for depilatory action. In this study, water was used as a diluent and emulsifier. CR, HA and PS were selected based on its traditional knowledge. *Cyperus rotundus* and pumice stone possess hair removal property, and *Hemigraphis alternata* possesses good wound healing properties (discussed in chapters 1 and 2). The prepared nanoparticles were also evaluated for their depilation efficiency due to their excellent biological activities and simple way of plant-mediated synthesis.

3.10.2 Preparation of depilatory cream using CRa, PS, HAa, CRaNP and PSaNP

A simple grinding method was used to prepare depilatory creams. Cetyl alcohol (CA) and Multani mitti (MM) was weighed according to the percentage mentioned in **Table 8**. Both were ground using mortar and pestle for 5 minutes. The samples (CRa, PS and HAa) were added to this mixture and ground well. Added the required amount of water (W), Urea (U) and NaOH (SH) in that mixture. The mixture was mixed well until it becomes homogenized and prepared creams were stored in plastic container tightly to avoid quick drying and interruption of moisture. The constituents used in all preparation were represented as Constant Mixture (CM (CM- CA, MM, U, SH and W)) and their percentage denoted as CM_X and it is given in **Table 8**.

Gold nanoparticles were prepared using CRablank and PSablank by mentioned methods in 3.4.3. The prepared nanoparticle was dried under vacuum and then weighed. Selected CRablank and PSablank embedded gold nanoparticles (1 mg) were dispersed in distilled water for 15 min and were incorporated during cream preparation by replacing CRa, PS and HAa.

From the preliminary studies, the percentages mentioned in **Table 8** were used in the preparation. The percentage of urea and sodium hydroxide was fixed by treating 1N NaOH (in the ratio of 1, 2, 3, 4 and 5) with 1N urea solution (1, 2, 3, 4 and 5). With an increase in the concentration of NaOH and urea, the hair weakens faster. This effect was noted by visually. The ratios of 1:1, 1:5 and 5:1 (The ratio represented by Urea:NaOH) showed a faster effect. Hence, the minimum ratio of 1:1 (Urea:NaOH) was taken in this study.

Table 8. The details of depilatory cream preparation using CRa, PS, HAa, CRaNP and PSaNP

S. No	Sample code	Constituents					Constituents (%)					Weight of the cream (g)
1	$CAMM_2$	CA	MM	U	SH	W	7	7	4	4	76	10.2999
2	CAMMCRa	CM		CRa			CM_X			2		10.1576
3	CAMMPSb	CM		PS			CM_X			2		10.1406
4	CAMMCRPS	CM	CRa	PS			CM_X	0.5		1.5		10.1295
5	CAMMCRPSHA	CM	CRa	PS		HAa	CM_X	0.6		0.9	0.3	10.1204
6	CAMMCRaNP	CM		CRaNP			CM_X			0.0043		10.2288
7	CAMMCRaNP(A)	CM		CRaNP			CM_X			0.017		10.0698
8	CAMMPSaNP	CM		PSaNP			CM_X			0.0043		10.2286

3.11 Testing of prepared depilatory creams

The prepared samples were tested using simple laboratory procedure. The commercially available creams also tested to compare the results.

3.11.1 Test for colour, odour and pH

The colour and odour of prepared samples were noted physically. The toxicity of the ingredients was analyzed before testing the odour and the samples odour were tested by holding the sample container 60 mm away from the nose. Prepared cream samples (500 mg) were dispersed in 50 mL distilled water **(Abhay *et al*., 2014)** by sonication for 5 min. The pH of prepared samples was noted using pH meter (HANNA pHep HI98107). The pH meter was initially calibrated using standard buffer solutions (pH 9.2 and 4).

3.11.2 Test for Loss on Drying (LOD)

Prepared samples (500 mg) are taken in a pre-weighed beaker and heated in a water bath (95°C) for 15 min. After that, the samples were desiccated for 15 min to avoid the absorption of moisture in hot condition. The final weights of the samples were taken, and the triplicate reading was noted. The percentage of LOD was calculated using **Equation 2.**

$$\text{LOD (\%)} = \frac{\text{Weight after drying}}{\text{Weight before drying}} \text{ X } 100 \qquad \text{………… Equation 2}$$

3.11.3 Spread-ability Test

Spread-ability was measured based on the methodology given by **Milla Gabriela *et al*. (2016)** with required modification. Initially, glass plates of 4x4 cm were pre-cleaned with absolute ethanol. Diameter (1 cm) was marked and drawn the circle on one side of the glass plate A. Accurately weighed the prepared samples (250 mg) and were placed inside the circle. Another unmarked glass plate B was placed on the top of the glass plate A without pressing and this glass sandwich allowed to one minute. The diameter of the cream travelled from the initial was noted and triplicate results were carried out.

3.11.4 Tests for stability

3.11.4.1 Centrifugation test

All the samples (250 mg) were taken in 1.5 mL centrifuge vials and centrifuged for 15 min in 3500 rpm. The separation of the composition was observed visually.

3.11.4.2 Stability on storage

All the samples (1g) were taken in plastic vials and kept in refrigerator (15°C) and room temperature (30°C). Every ten days (for 30 days) intervals, the colour, odour and appearance were noted visually.

3.11.5 EDS analysis of prepared depilatory samples

The presence of heavy metals and toxic substances was analyzed using EDS analysis. The samples were coated on glass plates and dried overnight in a vacuum desiccator and then it was analyzed.

3.12 *In vitro* depilatory evaluation of prepared creams using Fabricated Depilation Tester (FDT)

Based on section 3.9, the commercially available depilatory creams Std (V), Std (F) and the prepared depilatory creams (5 mg) was coated (1 cm/10 cm) on hair samples. The test was carried out in FDT. The average thickness of the hair is 0.024 mm ± 0.0091. The time of depilation was noted using a stopwatch and triplicate results were carried out.

3.12.1 Evaluation of cooling and room temperature effect on prepared depilatory samples

The cooling and room temperature effect was noted for prepared depilatory samples. All the samples (1g) were taken in plastic vials. The samples were kept in refrigerator (15°C) and room temperature (30ºC). Every ten days (for 30 days) intervals, the depilation time was noted.

3.12.2 Surface analysis of damaged hair samples using prepared depilatory creams

Depilatory creams treated and untreated hair samples (for comparison) were cut into small pieces. The creams present in treated hair samples were removed. The changes and keratin structure degradation were noted in surface morphological analysis with different magnifications.

3.13 Biological activities of prepared depilatory samples

Prepared depilatory samples were tested for antimicrobial, antioxidant and cytotoxicity activities. The methodology adopted for this study are given below.

3.13.1 Antimicrobial activity of prepared samples

Antimicrobial activity is one of the important parameters for every cosmetic product. In this study, the antibacterial and antifungal activity of prepared samples was carried out. Agar-well diffusion method was performed for this study. Different types of human pathogens (bacteria) such as gram-negative (*Escherichia coli* and *Salmonella paratyphi*) and gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) was used in antibacterial activity. In the antifungal activity, *Aspergillus fumigates* and *Aspergillus flavus* was used.

3.13.1.1 Sample preparation

The weight (100 mg/mL) of prepared samples (CAMM$_2$, CAMMCRa, CAMMPS$_b$, CAMMCRPS, CAMMCRPSHA, CAMMCRaNP, CAMMCRaNP(A) and CAMMPSaNP) was taken. The samples were completely dispersed for half an hour using sonication. Ciprofloxacin and Ketoconazole was used as antibacterial and antifungal standard respectively.

3.13.1.2 Evaluation of antibacterial activity using Agar-well diffusion method

The initial preparation for antibacterial activity was caried out by procedure mentioned in 3.8.1.2. Different volumes (150, 200 and 250 µL) of each samples (40 µL) were tested. The Petri dishes were incubated for 12h at 37ºC and the zone of inhibition was measured.

3.13.1.3 Evaluation of antifungal activity using Agar-well diffusion method

The Agar solution was prepared by treating Rose Bengal Agar (in the ratio of three) and Agar type II (in the ratio of one) in distilled water (300 mL). The mixture was sonicated for 5 min and micro-waved for 15 min. The required amount of agar solution was prepared. The agar solution was poured into sterilized Petri dishes, and it was kept until solidify. The required number of holes (0.5 cm) was created. The fungal strains (*Aspergillus flavus* and *Aspergillus fumigates*) were swabbed on the Petri dishes and samples (150, 200 and 250 µL) were added to it. The diameter zone of inhibition (mm) was measured after 48h.

3.13.2 Evaluation of antioxidant activity using DPPH radical scavenging activity

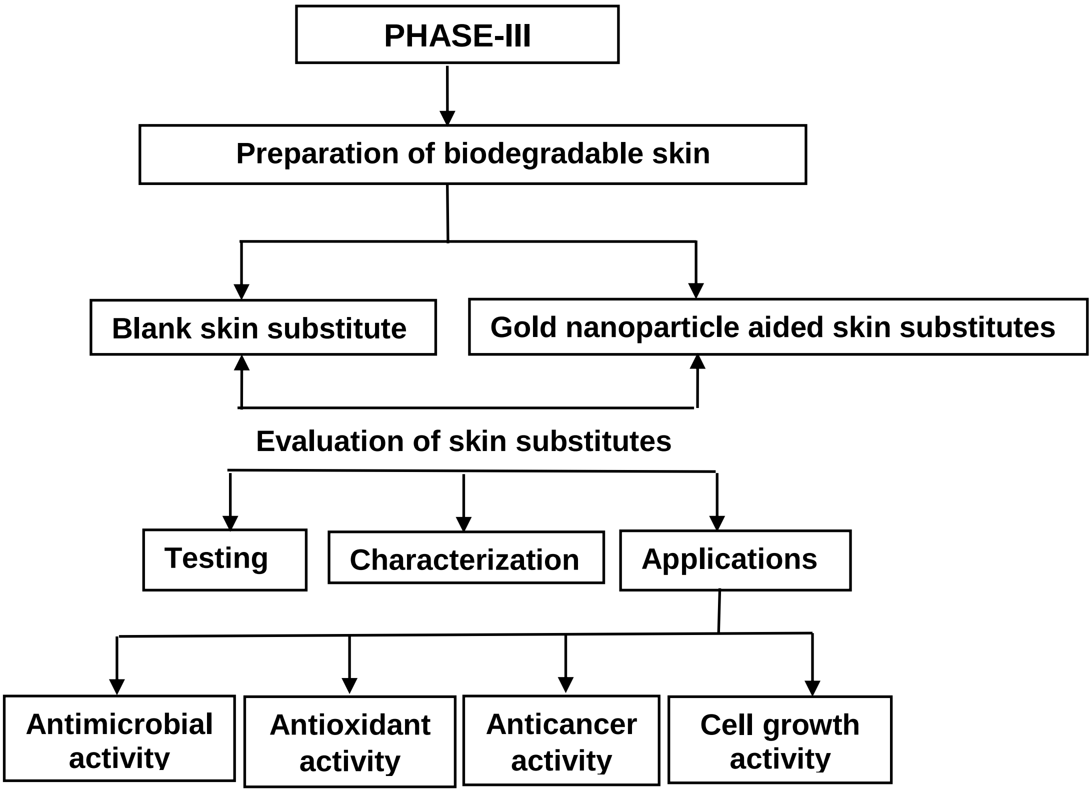
DPPH radical scavenging activity of prepared depilatory samples was carried out. The prepared samples (0.5, 1.5, 2.5, 3.5, and 5 mg/mL) was dispersed using sonication. DPPH solution (0.3 mM) was prepared by dissolving DPPH (3 mg) in Methanol (25 mL). Then the samples was treated with DPPH (0.3 mM) solution and incubated for 30 min in dark conditions. The absorbance of the sample was noted using a photoelectric colorimeter at the wavelength of 517 nm. The percentage of radical inhibition was calculated using **Equation 3**.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of DPPH in methanol} - \text{Absorbance of sample}}{\text{Absorbance of DPPH in methanol}} \times 100 \qquad \text{……… Equation 3}$$

3.13.3 Cell viability/cell toxicity activity of prepared samples

Cytotoxicity of prepared depilatory samples was evaluated using human skin cancer (A375 cells) and normal cell line (HEK-293). The cell viability was calculated by treating prepared samples (50, 100, 150, 200 and 250 µL) with MTT assay. The apoptotic activity using AO/EtBr and PI staining was carried out based on the IC_{50} value of the samples. The detailed procedures of this study is given in **Appendix I**. Based on the availability of human skin cancer cell lines, A431 cell lines were used in Phase I and A375 cell line were used in Phase II and III.

The overview of phase III are given in the following flow chart.



3.14 Preparation of constituents for fabrication of skin substitutes

3.14.1 Selection of constituents

In the present study, naturally available biopolymers gelatin, sodium alginate and chitosan were used. Tea dust contains valuable antioxidant agents **(Sarah and Joshua, 2011)** to help in maintaining the antioxidant activity of the skin substitutes. Fenugreek is one of the food ingredients with more medicinal values and it is also used in skincare application. It contains more adhesive and emulsifying property **(Sajad and Kumar, 2018)**. In this regard, to enhance the adhesive property of the skin substitute, the extraction of adhesive fenugreek mucilage was carried out based on **Nayak *et al*., 2013** with some modifications. Based on the preliminary studies, constituents, and their ratios to prepare the skin substitutes were chosen.

3.14.2 Preparation of tea extract

Tea dust (5g) was refluxed (1h) with distilled water (200 mL) at 80–90°C. The solution was filtered using the cotton plug and dried in a vacuum desiccator, followed by weight taken. The aqueous extract of tea dust (1.5g) was made up into 250 mL in a standard measuring flask and kept in the refrigerator for further study.

3.14.3 Preparation of fenugreek mucilage

Fenugreek samples (**Figure 4a**) were purchased from a local shop in Udumalpet, Tamilnadu, India. The dried seeds were made into a fine powder. Powdered fenugreek (100g) was soaked overnight with distilled water (2L) and then refluxed for 4h at 60–70°C. The refluxed mixture was filtered using a sieve and then a cotton cloth. Ethanol (2L) was added into the filtrate and it was allowed for 15 min to settle. The white colour mucilage appeared at the bottom of the container and is given in **Figure 4b**. After removing the solvent, the dried fenugreek mucilage powder was stored.

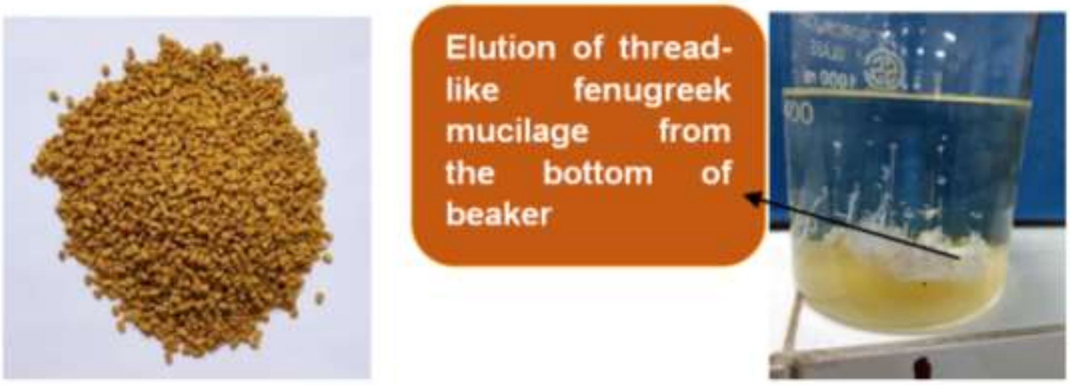


Figure 4a. Photograph of fenugreek seeds

Figure 4b. Photograph of fenugreek mucilage

Figure 4a and b. Extraction of mucilage from aqueous extract of fenugreek seeds using ethanol

3.14.4 Preparation of glycerol and water mixture

Glycerol and water were taken in the ratio of 1:3 respectively. The mixture was sonicated for 15 min and then stored in a standard measuring flask for future use.

3.15 Preparation of gold nanoparticles-aided skin substitute

3.15.1 Preparation of gold nanoparticles

CRablank (10 mL) and HAablank (10 mL) were treated with auric chloride (10 mL) and kept in the domestic microwave oven and room temperature, respectively. The synthesized gold nanoparticles were dried and weighed using a pre-weighed container. Furthermore, it was sonicated and homogenized with distilled water (20 mL) for 15 and 30 min, respectively.

3.15.2 Synthesis of gold nanoparticles-aided skin substitutes

Granules of gelatin, dried fenugreek mucilage and sodium alginate were separately dissolved with the required amount of water (**Table 9**). Chitosan powder was dissolved in 2% acetic acid by stirring 10 minutes. The sodium alginate solution was added to the chitosan under magnetic stirring conditions and then added gelatin and fenugreek mucilage.

The mixture was homogenized for 15 min, added tea extract and glycerol-water and filtered using a sieve. Then it was finally stirred and heated using a hotplate for 30 min at 1000–1050 rpm. The prepared plant-embedded gold nanoparticles (5, 10, and 15 mg/mL) were added to the mixture. Then it was stirred for 5–6h until the mixture reduces to half. The hot mixture was poured onto the heat-resistant silicon rubber mat and spread evenly by gentle shaking. Then it was dried for 24h and stored in a Ziplock cover for further study. The nanoparticle untreated samples are known as blank.

Table 9. The formulation details of biodegradable skin substitutes

H(g)-Water for glycerol; **H(c)**-Acetic acid for chitosan; **H$_{(FS)}$**-Water for fenugreek seeds; **H$_{(a)}$**-Water for sodium alginate; **G**-Gelatin; **C**-Chitosan; **FS**- Fenugreek seed mucilage; **A**-Sodium alginate; **Y**-Glycerol; **GT**-Green Tea

S. No	Sample code	Constituents	Quantity	Initial Colour	Final colour
1	SLFgNP$_0$	G+C+FS+A+Y+GT+ H(g) + H(c) + H$_{(FS)}$ + H$_{(a)}$	0.1g+0.3g+0.3g+0.1g+ 5 mL+3 mL+10 mL+20 mL+20 mL+10 mL	Yellow	Yellow
2	SLFgCRaNP$_1$	SLFgNP$_0$ + CRaNP	SLFgNP$_0$+5 mg/mL		Yellowish Purple
3	SLFgCRaNP$_2$		SLFgNP$_0$+10 mg/mL		
4	SLFgCRaNP$_3$		SLFgNP$_0$+15 mg/mL		
5	SLFgHAaNP$_1$	SLFgNP$_0$ + HAaNP	SLFgNP$_0$+5 mg/mL		Yellowish Brown
6	SLFgHAaNP$_2$		SLFgNP$_0$+10 mg/mL		
7	SLFgHAaNP$_3$		SLFgNP$_0$+15 mg/mL		

3.16 Testing of prepared skin substitutes

Simple laboratory testing procedures were carried out for prepared skin substitutes and triplicate results were taken. The mean and standard deviation was taken for the results using Microsoft Excel 2007.

3.16.1 Measurement of colour, thickness, texture and stability

The thickness of the prepared samples was measured in 10 different places of skin substitutes using a screw gauge. The colour and texture of the skin substitutes were noted visually. The microbial contamination of skin substitutes was analyzed for two years. The samples (10×10 mm) were soaked in distilled water (5 mL) and kept at room temperature. The stability, appearance, and microbial contamination of skin substitutes were analyzed visually for 2 years. At the end of every week, the water was changed.

3.16.2 Mechanical properties of prepared skin substitutes

The tensile and strain properties of the prepared skin substitutes (10 mm x 150 mm) were carried out according to test standard ISO 1924-1/2 in Zwick /Roell Z010 Tensile meter. The samples were operated at 200 mm/min, and the test speed is 5 N/m.

3.16.3 Moisture content test

The moisture content were carried out based on **Wu *et al*., 2015** and **Yao *et al*., 2017**. Initially, the samples (10 x 20 mm) were weighed and kept in a hot air oven for 24h at 105°C. The hot samples were kept in a desiccator for two hours and then weighed. The percentage of moisture content of the samples is obtained from **Equation 4**.

$$\text{Moisture content } (\%) = \frac{W_a - W_b}{W_a} \times 100 \quad \text{................ Equation 4}$$

W_a - Weight of prepared skin substitutes before drying

W_b - Weight of prepared skin substitutes after drying

3.16.4 Water uptake and Water ageing analysis

The initial weight of prepared samples (10 × 10 mm) was taken and then soaked in 5 mL distilled water. Every 10 min (10, 20, 30, 40, 50, and 60 min) intervals, the wet and dried weight of the samples was taken at room temperature. The percentage of water uptake and water ageing was calculated using **Equations 5** and **6**, respectively.

$$\text{Water uptake } (\%) = \frac{W_a - W_b}{W_b} \times 100 \quad \text{................ Equation 5}$$

W_a - Weight of prepared skin substitutes after soaking

W_b - Weight of prepared skin substitutes before soaking

$$\text{Water ageing } (\%) = \frac{W_a - W_b}{W_b} \times 100 \quad \text{................ Equation 6}$$

W_a - Weight of prepared skin substitutes after soaking

W_b - Weight of prepared skin substitutes after drying

3.17 Characterization of the prepared skin substitutes

3.17.1 FTIR analysis

The active functional groups present in the skin substitutes were identified using FTIR (MIRacle 10 Shimadzu). The samples (10x10 mm) were scanned in the range of 4000-400 cm^{-1}.

3.17.2 Thermo Gravimetric Analysis

Prepared skin substitutes were analyzed for their thermal degradation properties under a nitrogen atmosphere, and the temperature was taken in the range from room temperature to 800°C.

3.17.3 3D Optical Profilometric Analysis

Three-dimensional structural views of prepared skin substitutes were analyzed using 3D Optical Profilometer (Zeta-20). The prepared samples (10x10 mm) were kept under the optical profiler, and the 3D images were taken from the samples. The average roughness and texture were noted at step size 100-350 µm and the view field of 95x71 µm.

3.17.4 Surface Morphology Analysis

The surface morphology of prepared skin substitutes were analyzed in FESEM and elemental analysis were carried out using EDS. The elemental distribution of the samples were also noted by mapping.

3.18 Biological activities of prepared skin substitutes

Prepared skin substitutes were tested for their antifungal, antioxidant and cytotoxicity activities. Cell growth and cell attachment in prepared skin substitutes also evaluated. The methodologies adopted for this study are given below.

3.18.1 Antioxidant activity of prepared skin substitutes

Antioxidant activity of skin substitutes was carried out using the DPPH assay as per the reported method **(Kavoosi *et al*., 2013)** and with some modifications. Prepared skin substitutes were cut into 10×10 mm and added methanol. It was then sonciated for 5 min. Then the sample mixture was treated with DPPH (0.3 mM) in dark conditions. The DPPH-treated samples were incubated for 30 min in dark conditions. The absorbance of the sample was noted using a photoelectric colorimeter at a wavelength of 517 nm. Then the percentage of radical inhibition was calculated.

3.18.2 Antifungal activity of prepared skin substitutes

The disk diffusion method was used to analyze the antifungal activity of prepared skin substitutes. All the apparatus was washed with a soap solution and water. Then it was dried in a hot air oven for 1h. The Petri dishes were again cleaned with ethanol

(99.9%) and then UV sterilized (30 min). Rose Bengal and agar type II were sonicated with distilled water (300 mL) for 15 min, followed by microwave for 10 min. The hot solution was poured into cleaned Petri dishes and kept for 30 min. The fungal strains were swabbed onto the agar plate using a cotton stick. Prepared skin substitutes (0.6×0.6 cm) were placed on the Petri dishes using sterilized forceps and placed in a non-disturbing area. After 2 days, the fungal inhibition zone was measured. Commercially available ketoconazole was used as a standard.

3.18.3 Anticancer activity of prepared skin substitutes

3.18.3.1 Evaluation of MTT assay

The cell toxicity of prepared skin substitutes was tested against A375 and HEK-293 cell lines. The samples were homogenized for one hour, resulting in the samples were dispersed into fine particles. It was further sonicated for half an hour, and then the IC_{50} value was evaluated by MTT assay. The samples were treated in the volume of 50, 100, 150, 200, and 250 µL with cell lines, and a defined procedure was followed (**Appendix I**). The OD value was measured at 620 nm using ELISA multiwell plate reader. The apoptosis cell death was observed for skin substitutes by treatment with PI and AO/EtBr dye mixture. The cell death was monitored using a fluorescence microscope.

3.19 Cell attachment and cell growth activity studies

Cell attachment and cell growth activity was carried out based on **Ezati *et al.*, 2018** with some modifications. The HEK-293 cell lines were maintained in Dulbecco’s Modified Eagles Medium (DMEM) and supplemented with 2 mM L-glutamine, BSS, nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), glucose (1.5 g/L), HEPES (10 mM), and fetal bovine serum (10%). Finally, Penicillin and Streptomycin were adjusted to mL/L. Then the cells were incubated with CO_2 (5%) in a humidified CO_2 incubator. The cells were cultured in 48 well plates for two days in 75% confluence. The well plate contained 1×10^4 cells/well, and the prepared samples were placed in half the portion of the well. The cell growth and cell attachment on the skin substitutes were noticed using a microscope.