3

MATERIALS AND METHODS

3.1 General

Doubly distilled water was used throughout the studies. All glassware used for the studies was thoroughly washed with soap water followed by water washing, air drying and swabbing with acetone. Necessary biosafety precautions are followed while performing the biological studies

3.1.1 Chemicals

All the chemicals and solvents used for the study are of AR grade. Hydrogen tetrachloroaurate trihydrate ($HAuCl_4.3H_2O$), Silver nitrate, Muller Hinton agar, Phosphate buffer pH ~7.2, K_4 $[Fe(CN)_6]$ (HI-MEDIA) are used in this study. Melamine, Graphite powder, Cetyl alcohol and $ZnSO_4.7H_2O$ were purchased from Loba chemicals. Commercial Graphene oxide, Calf thymus DNA, Tris HCl was purchased from Sigma Aldrich. Glycerol was purchased from Hipure.

3.1.2 Instruments and equipments

Listed below are the instruments and equipments used for the current research work

- Electronic balance (Shimadzu ATY 224)
- Water bath (SAFIRE India Pvt. Ltd.)
- Ultra sonic bath (Kinglab/KLDUC-6L);
- Ultrasonic probe Homogenizer (VCX 500 vibracell)
- pH meter (Eutech pH tutor I)
- Turbidity meter (Systronics 132)
- Magnetic stirrer (REMI 1MLH)
- Microwave oven (LG ECN:MS1947C/01)
- Hot air oven (SELEC TC303)
- Bio spec Nano spectrophotometer (Shimadzu/ 206-26300-48)
- FT-IR spectrophotometer (Shimadzu IR affinity1)
- X-ray diffractometer (X'pert pro, PANALYTICAL)
- Thermo Gravimetric Analyzer (EXSTAR/6300 instrument)
- Raman spectrophotometer (RE- 3000 QE instrument)
- Zeta potential (Malvern Instruments Ltd.)
- Field Emission Scanning Electron Microscope with EDX (TESCAN MIRA3 XMU)
- 3D Optical Profilometre (Zeta 20)
- Plasma chamber (Ss 304 Vacutech System)
- ELISA (Thermo Multi - skan EX, USA)
- Electrochemical workstation (AMETEK PARSTAT MC-1000)

3.2 Bioreductants used in the study

TB dry fruit was collected from the local herbal shop in Palakkad district, Kerala, India and certified by department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore. Various Indian rice grains, toor dhal, chana, were collected from local shops in Coimbatore district. Rice varieties from Thailand, Indonesia and Boston were also utilized in this study. Commercial *Garcinia combogia* capsules were purchased from the local shop in Coimbatore. Free edges of *Corpus unguis* of female aged 28 (which was discarded as waste) was utilized in this study. The free edge of the *Corpus unguis* does not require human ethical clearance as waste tissues materials are utilized in this study (**Petrini, 2012**). The methodology of the studies carried out are categorized and listed below

- **Preparation of bioreductants and analysis**
  - Extraction of plant materials, conventional washing of cereals/pulses and preparation of nail samples
  - Phytochemical screening of plant extracts
  - Nutritional value evaluation of cereals and pulses washed water
  - Elemental analysis and physico-chemical studies of cereals/pulses washed water and elemental analysis of HN28
- **Physico-chemical synthesis of metallic and non-metallic nanoparticles**
  - Preparation of stock solutions
  - Physico-chemical synthesis of GNPs
    - Synthesis of GNPs at room temperature
    - Synthesis of GNPs by solar irradiation
    - Synthesis of GNPs by sonication
    - Synthesis of GNPs by microwave heating
  - Synthesis of SNPs by solar irradiation method
  - Synthesis of ZnONPs by sol-gel method
  - Synthesis of reduced graphene oxide by reflux method
- **Analytical Characterization of nanoparticles**
  - Ultraviolet (UV)-Visible spectroscopic analysis
  - Fourier Transform Infra-red spectroscopic (FT-IR) analysis
  - X-ray Diffraction (XRD) analysis
  - Zeta potential analysis
  - Field Emission Scanning Electron Microscopic (FE-SEM) analysis and Energy Dispersive X-ray spectroscopic analysis (EDS)
  - Raman spectroscopic analysis
  - Thermo Gravimetric Analysis (TGA)
- **Applications of synthesized nanoparticles**
  - Bactericidal activity of TB-GNPs and TB-SNPs against pathogens
    - Strain and culture media preparation
    - Test procedure for anti-bacterial testing
    - Minimum Inhibitory Concentration (MIC) studies
  - Anti-bacterial earphone buds coated with TBOSNP

- ❖ Bactericidal activity of GNPs and SNPs synthesized from cereals and pulses washed water
- ❖ Anti-cancer activity of GNPs and SNPs from cereals and pulses washed water against A375 skin cancer cell lines
    - Calculation of cell viability
- ❖ DNA binding studies of GNPs and SNPs
    - Spectrophotometric analysis of DNA-nanoparticle interaction
- ❖ *Allium cepa* root tip toxicity assay for GNPs
- ❖ Sun screen lotion formulation incorporating nanoparticles
    - Preparation of lotion formulation incorporating HN28Au
    - Preparation of lotion formulation incorporating ZnONPs
    - Physico-chemical analysis of lotion formulations
        - ❖ Stability test
        - ❖ Spreadability test
        - ❖ Colour and pH test
        - ❖ *In vitro* occlusivity test
    - Bactericidal activity of lotion formulations
    - Spectrophotometric determination of sun protection ability of lotion formulations
- ❖ Nanoswitching/sensing application of nanoparticles
    - Colorimetric sensing of ammonia by TBPGNP
    - Colorimetric sensing of cyanide by GCAu
    - Electrochemical cyanide sensing by GCAu@CPE
        - ❖ Fabrication of GCAu@CPE
        - ❖ Electrochemical cell setup and analysis
- Electrochemical cyanide sensing by low cost CPE in corn sticks
- Analytical sensing of melamine leached out from melamine tableware
    - ❖ Preparation of stock solutions
    - ❖ Melamine leaching studies
    - ❖ FT-IR analysis of leached melamine samples
    - ❖ Comet assay determination of DNA damage by leached melamine samples
- Electrochemical melamine sensing by BJRGO@CPE
    - ❖ Fabrication of BJRGO@CPE
    - ❖ Stability and reusability test for fabricated electrodes
    - ❖ Electrochemical cell setup for melamine sensing

The goal of this research is to synthesize metallic and non-metallic nanoparticles using bioreductants and explore selected applications of the synthesis nanoparticles.

3.3. Preparation of bioreductants and analysis

3.3.1 Extraction of plant materials, conventional washing of cereals/pulses and preparation of nail samples

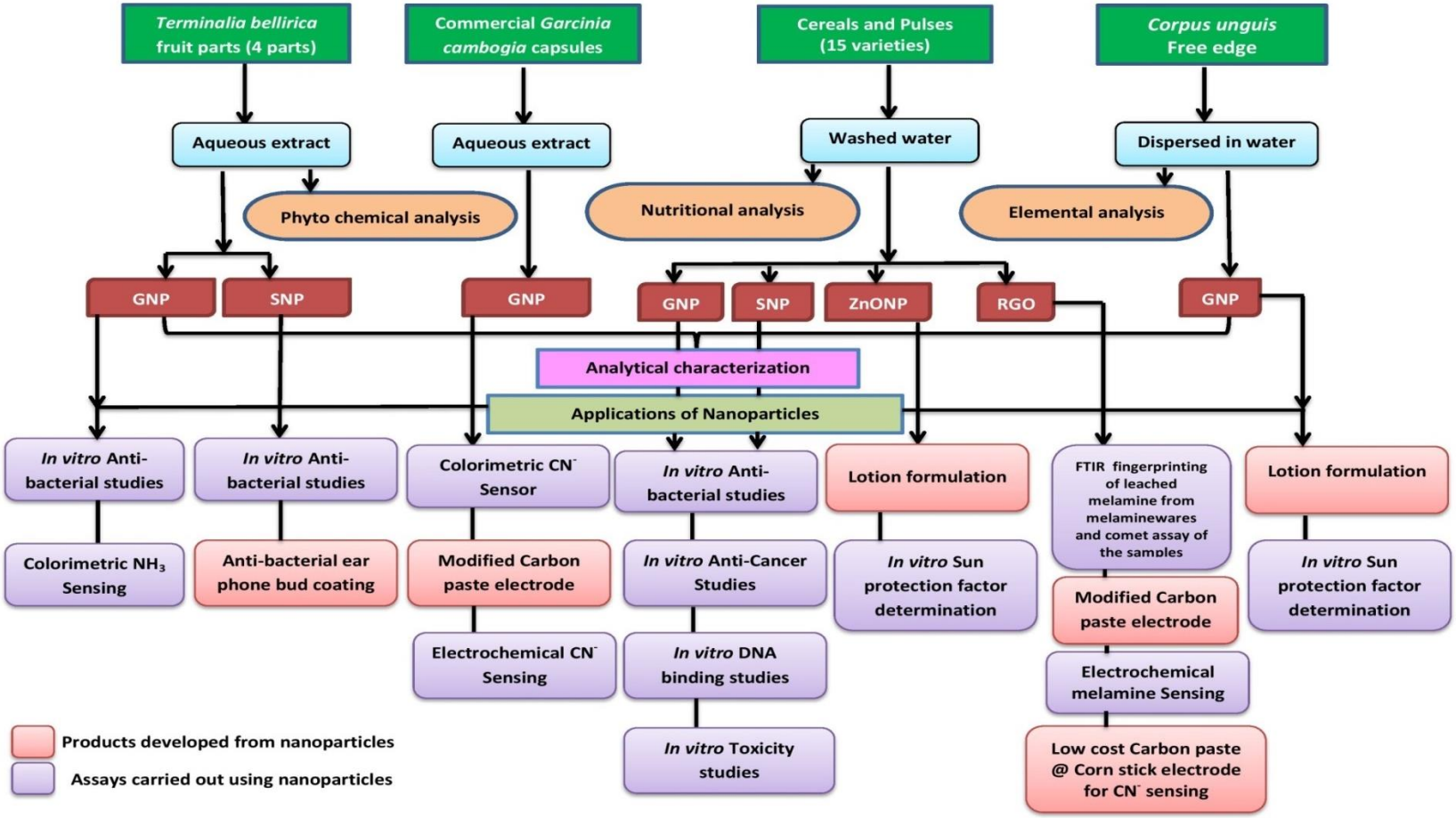
The details of the bioreductants used in this research work are listed in **Table 5.** From the dry fruit of *Terminalia bellirica the* epicarp, mesocarp and seed were separated and powdered well. Whole fruits were powdered as such. To each fruit part (1g), 40 mL distilled

water was added and kept under steam bath for 20 min. Similar procedure was adopted for GC capsules. Extracts were filtered using a cotton plug and sonicated again for 10 min and used for the synthesis of NPs. The cereals and pulses washed with distilled water (1:5 ratio) was filtered using cotton plug. Hithereto in this thesis, this water is referred as washed water. This washed water was refrigerated and used within a week. The collected *Corpus unguis* was sonicated in water and the obtained dispersed water was used in this study.

Table 5. List of bioreductants utilized for the study

S.No.	Bioreductants	Sample code	Common Name	Used part
1	*Terminalia bellirica*	TBOS	Bahera	Dry Fruit; Epicarp
2	*Terminalia bellirica*	TBIS	Bahera	Dry Fruit; Mesocarp
3	*Terminalia bellirica*	TBS	Bahera	Seed
4	*Terminalia bellirica*	TBP	Bahera	Whole dry fruit
5	*Oryza sativa*	IBoRW	Parboiled Rice	Washed water
6	*Oryza sativa*	IRaRW	Raw Rice	Washed water
7	*Oryza sativa*	IRRW	Red Rice	Washed water
8	*Oryza sativa*	IBrRW	Broken Rice	Washed water
9	*Oryza sativa*	IBRRW	Broken Red Rice	Washed water
10	*Oryza sativa*	IBaRW	Basmati Rice	Washed water
11	*Triticum aestivum*	IWW	Punjab Wheat	Washed water
12	*Fagopyrum esculentum*	BuWW	Buck wheat	Washed water
13	*Pennisetum glaucum*	BJWW	Bajra	Washed water
14	*Cicer arietinum*	WCWW	White chana	Washed water
15	*Cajanus cajan*	TDWW	Toor dhal	Washed water
16	*Oryza sativa*	InB	Brown rice (Indonesia)	Washed water
17	*Oryza sativa*	InW	Raw rice (Indonesia)	Washed water
18	*Oryza sativa*	ThW	Raw rice (Thailand)	Washed water
19	*Oryza sativa*	BoW	Raw rice (Boston)	Washed water
20	*Garcinia cambogia*	GC	Pot tamarind	Capsules
21	*Corpus unguis*	HN28	Human nail	Free edge

Flow chart representation of work carried out in the entire study



3.3.2 Phytochemical screening of plant extracts

Aqueous extracts of TB was subjected to phyto chemical screening to explore the secondary metabolites present in it. The test procedures were verified and followed according to the reported procedures (**Raaman, 2006**; **Jayanthi *et al*., 2012**).

3.3.3 Nutritional value evaluation of cereals and pulses washed water

Determination of active constituents in the cereals and pulses washed water were done by nutrient analysis. The nutrients present in the cereals and pulses washed water viz., Vitamin, fat, protein, carbohydrates, moisture and total ash were analyzed quantitatively adapting standard procedures previously reported. The analysis was certified by Alpha labs, Coimbatore, India (NABL accredited **ISO 9001 certified** laboratory) (**Appendix 1**).

3.3.4 Elemental analysis and physico-chemical studies of cereals/pulses washed water and elemental analysis of HN28

Elemental analysis by EDS was executed by coating about 4µL washed waters directly on to a conductive double sided carbon tape fixed on the Al stub. The EDS analysis was done for gold sputtered samples. The active elemental composition was determined from EDS analysis (Apex software). Similar procedure was adopted for the elemental analysis of HN28 by EDS. The physico-chemical factors such as turbidity, pH was determined for the cereals and pulses washed water. For turbidity studies Formazine 400 NTU was used as a standard. The pH studies were carried out in digital pH meter which was pre calibrated.

3.4 Physico-chemical synthesis of metallic and non-metallic nanoparticles

The synthesis of GNPs and SNPs was carried out using bioreductants under various physico chemical approaches. The adapted physico-chemical methods are widely used for the synthesis of NPs. Room temperature (RT) ($25^{o}C$-$30^{o}C$), Microwave (M), Sonication (So), Solar irradiation (Su).

3.4.1 Preparation of stock solutions

The stock solutions for $HAuCl_4$ (3mM) and $AgNO_3$ (3mM) was prepared using required quantity of respective metallic salts and dissolved in distilled water. $ZnSO_4.7H_2O$ (1M) was prepared in distilled water. Commercial graphene oxide dispersed in water (2mg/mL) was used as such for the study.

3.4.2 Physico-chemical synthesis of metallic nanoparticles

3.4.2.1 Synthesis of GNPs at room temperature

Biosynthesis of GNPs were done by taking equal volume of $HAuCl_4$ (3mM) and equal volume of bioreductants (TBOS, TBIS, TBS, TBP, IBoRW, IRaRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW) and allowed to reduce at room temperature. The reaction was optimized by fixing the volume of $HAuCl_4$ constant (100µL)

and varying the volume of bioreductants in the ratio 1:1, 1:2, 1:3, 1:4 and 1:5. The formed GNPs were identified by the change in colour. Time of formation was also noted.

3.4.2.2 Synthesis of GNPs by Solar irradiation

$HAuCl_4$ (100µL) was mixed with varied volume of bioreductants (IBoRW, IRaRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW, HN28) in the ratio 1:1, 1:2, 1:3, 1:4 and 1:5 and placing them under sunlight. The formed GNPs were identified by the change in colour. The time required for the bioreduction process is also recorded.

3.4.2.3 Synthesis of GNPs by Sonication

The synergistic effect of sound waves on the bio reduction of $HAuCl_4$ was monitored. The reaction was monitored by mixing equal volume of $HAuCl_4$ constant (100µL) and varying the volume of bioreductants (IBoRW, IRaRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW) in the ratio 1:1, 1:2, 1:3, 1:4 and 1:5 and placing them in ultrasonic bath, with time of sonication set for 5 min in the equipment. The formed GNPs were identified by the change in colour. The time required for the bioreduction process is also recorded.

3.4.2.4 Synthesis of GNPs by Microwave heating

$HAuCl_4$ (100µL) was mixed with varied volume of bioreductants (IBoRW, IRaRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW, InB, InW, ThW, BoW, GC) in the ratio 1:1, 1:2, 1:3, 1:4 and 1:5 and placing them in microwave oven. The formed GNPs were identified by the change in colour. The time required for the bioreduction process is also recorded.

3.4.3 Synthesis of SNPs by Solar irradiation method

Reduction of $AgNO_3$ was carried out by bioreductants (TBOS, TBIS, TBS, TBP, IBoRW, IRaRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW, InB, InW, ThW, BoW). Volume of $AgNO_3$ (3mM) was kept constant (100µL) and reducing agents were taken in the ratio 1:1, 1:2, 1:3, 1:4 and 1:5. This mixture was exposed to sunlight. The formed SNPs were identified by the change in colour of the mixture. Time at which colour changes was recorded.

3.4.4 Synthesis of ZnONP by sol-gel method

The washed water IRaRW (50mL) was added to $ZnSO_4.7H_2O$ (50mL) drop wise and stirred continuously for 2h at 1400rpm. To this mixture about 50mL NaOH (0.45M) is added and pH is checked and ensured to be alkaline.

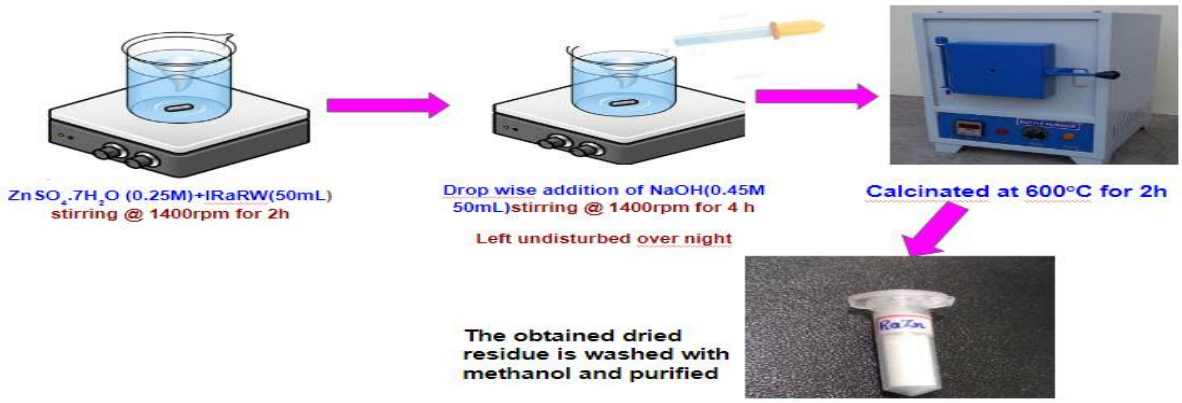


Figure 2. Schematic representation of synthesis of ZnO nanoparticles from cereals and pulses washed water

The stirring was continued for another 4h. This mixture is left undisturbed overnight. The precipitate is centrifuged and residue obtained is washed with distilled water and centrifuged again and dried. The residue is treated in muffle furnace (600^{o}C) for 2h and washed with methanol and air dried (**Figure 2**). The obtained powder is further used for characterization. Similar procedure is adopted for the synthesis of ZnONP with IBoRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, BJWW, WCWW, TDWW samples.

3.4.5 Synthesis of reduced graphene oxide by reflux method

BJWW (150ml) was refluxed with 100ml (2mg/ml) of graphene oxide for 6h at 80-90^{o}C. The colour of the solution turns black ensures the reduction of graphene oxide. The solution is sonicated well and centrifuged. The residue is dried to obtain reduced graphene oxide and utilized for further analysis. Similar procedure was adopted for the synthesis of reduced graphene oxide using IBoRW, IRRW, IBrRW, IBRRW, IBaRW, IWW, BuWW, IRaRW, WCWW and TDWW.

3.5 Analytical characterization of nanoparticles

Analytical characterization techniques help to determine the size, surface morphology and structural properties and ensure the nature of synthesized nanomaterials. Basic to high-end characterization techniques were adopted in this study and are detailed below.

3.5.1 Ultraviolet (UV)-Visible spectroscopic analysis

UV-Visible analysis is one of the preliminary analytical techniques adopted to ensure the formation of nanoparticles with the help of characteristic surface plasmon resonance band (SPR) particularly for gold and silver nanoparticles. The optical nature of the material can be determined using this technique, which will eventually help to focus any application with optical properties. The analysis was carried out in Bio-spec nano spectrophotometre in photometric mode with path length 0.7mm and wavelength ranges from 220nm to 800nm. All the synthesized nanoparticles were subjected to UV-visible analysis.

3.5.2 Fourier Transform Infra-red Spectroscopic analysis

The structural properties of the nano materials and functional group responsible for the reduction of metal ions to metal nanoparticles can be determined using FT-IR analysis. ATR-FT-IR with Lab solution software was utilized in this study. Samples were recorded in mid IR region of wave number (4000-400 cm^{-1}). The samples are recorded in % transmittance mode with parameters: 45 continuous scans, with resolution 16cm^{-1}; Happ-Genzel apodization was customized using the Lab solutions software. Background scan against atmospheric air was recorded. ZnONP and reduced graphene oxides synthesized were placed in solid form. Gold and silver nanoparticles in aqueous solutions are directly placed on the ATR accessory and recorded.

3.5.3 X-ray Diffraction analysis

The crystalline and amorphous nature of the synthesized nanomaterials was determined using X-ray diffractometer. Drop casted and air dried Gold and Silver nanoparticles over glass plates (2cm x 2 cm) are taken for analysis. RGO samples are directly spread over the sample holder and recorded. From the obtained 2θ values, crystallite size of the particle can be calculated by substituting the values obtained from spectra in Debye-Scherrer equation. The scanning parameters are Cu Kα radiation (λ=1.54060 Å) monochromatic filter operated at 45 kV and 30 mA over the range of 2θ=10°–80° with scanning rate 2º per min. Bragg's law $2dSin\theta=n\lambda$ is used to calculate crystallite size.

3.5.4 Zeta potential analysis

Zeta potential analysis is done to determine the surface charges and stability of the colloidal nanoparticles. The synthesized gold and silver nanoparticles in aqueous form are taken for analysis.

3.5.5 Field Emission Scanning Electron Microscopic (FE-SEM) analysis and Energy Dispersive X-ray spectroscopic analysis (EDS)

The surface morphological studies of the synthesized nanomaterials Gold, silver, Zinc oxide and reduced graphene oxides were analyzed using FE-SEM. All the samples were well sonicated in water and dried. The dried samples were coated over the double sided carbon tape fixed in the Al stubs. The samples were gold sputtered, this made samples conductive and free from non-conducting impurities if any. The electron voltage from 5kV to 30kV was applied to the samples. The elemental analysis of the samples was done using EDS instrument attached with Apex software. The elemental analysis helps to better understand the percentage of elements in the samples and their distribution over the surface of the nanomaterials.

3.5.6 Raman spectroscopic analysis

In order to determine the structural geometry, bonding and the layer thickness Raman spectroscopy analysis can be done. It is a non-destructive technique, which will not

alter the nature of the materials. The vibrational frequency responses of synthesized reduced graphene oxide materials were analyzed with resolution 6 cm^{-1} and excitation wavelength 785nm.

3.5.7 Thermo gravimetric analysis

The thermal stability and sensitivity of the synthesized reduced graphene oxide and Zinc oxide nanomaterials were determined using thermogravimetric analysis under inert N_2 atmosphere with 100mm/min. The samples are filled inside a pre cleaned alumina pan about 10mg and heated upto 1000^oC, temperature are increased at the rate of 20^oC/min. The masses were recorded as a function of time.

3.6 Applications of synthesized nano particles

The applications of synthesized metallic and nanoparticles in the field of pharmacology, biology, cosmetics and sensors are detailed below.

3.6.1 Bactericidal activity of TB-GNP and TB-SNP against pathogens

3.6.1.1 Strain and Culture media preparation

The bacterial activity of aqueous extracts of TB dry fruit parts, GNP capped with these extracts and standard (Amikacin 2ml/100mg) and tested against clinical pathogens following the standard procedures. Pathogens chosen are *A.pneumonia*, *Enterococcus faecalis* and *B.subtilis.*

The antimicrobial property of the synthesized TB silver nanoparticles and Ciprofloxacin (standard) was examined against clinical *Bacillus subtilis, K. pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Clinical microbial cultures were obtained from the Coimbatore, Tamil Nadu. The media for the microbiological test was made of Himedia sterile discs and Himedia antibiotics disc (Ciprofloxacin 5g) from Bombay, India. Himedia zone reader was used to assess the antibacterial activity. About 100 µL clinical *Bacillus subtilis, K. pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* organisms were inoculated individually in 5.0 mL sterile nutrient broth (NB) media and incubated at 37°C for 24h. To standardize the bacterial culture to 10^8 CFU/ml, 200 µL from the organisms' 24-hour fresh culture was poured into 30 mL sterile nutrient broth and cultured for 2-4 hours. TB SNP are separately dissolved in 1000 µL sterile water, from the stock solution under aseptic arrangements, 100 µL diluted formulation were added to Himedia sterile discs. Dried formulation discs are utilised for antibacterial activity and are entirely air dried under aseptic conditions.

3.6.1.2 Test procedure for anti-bacterial testing

To evaluate the efficacy of our formulations, we chose the well plate method for bacterial studies of TBGNP. To establish the inoculum, we employed nutrient broth media to cultivate three distinct microorganisms: *Acinetobacter pneumonia, Bacillus subtilis,* and *Enterococcus faecalis*. Subsequently, we autoclaved 7.6 grams in 100 milliliters Muller

Hinton agar media, generating a double-strength sterile solution. Using sterile cotton swabs, we inoculated the aforementioned microorganisms onto Muller Hinton agar plates then placed extracts of TB dry fruit parts, GNP, and Amikacin 100µL/250g into an agar well. After incubating the plates for 30 minutes to facilitate diffusion of the formulation into the agar plate, we proceeded to incubate them at 37 degrees Celsius for 24 hour. Finally, we employed a Himedia zone reader to assess the antibacterial activity.

The synthesized nanoparticles and Ciprofloxacin (5µg) were evaluated using disc diffusion method. *Bacillus subtilis, K. pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* inoculums prepared using sterile nutrient broth media. Mueller Hinton agar plates are inoculated with standardized inoculums (test microorganisms) using sterilized cotton swabs. Under aseptic circumstances, Ciprofloxacin (5g) discs and silver nanoparticles were applied to agar. Agar plates were incubated at 37^{o}C(24h) after being incubated at the refrigerator for 30 min to diffuse the formulation into the agar. Antibacterial activity is measured, using Himedia zone reader.

3.6.1.3 Minimum Inhibitory concentration studies

A micro-dilution approach was used to determine the Minimum Inhibitory Concentration (MIC) for the synthesized SNP and the corresponding controls. The bacterial cultures were fixed to an optical density (OD) of 0.22 at 595 nm using a spectrophotometer (OD595= 0.22) corresponding to 10^8 CFU/mL. About 10 mL inoculum of the aforementioned bacteria culture was placed in each well of a 96-well cell culture plate along with various doses of SNP and the corresponding controls. The plates were incubated at 37 °C for 24 hr. Each 96-well cell culture plate containing the treatment and the control cultures received an addition of around 100 µL of Tetrazolium stain (TTC) solution (0.5% w/v).Well plates were incubated at room temperature for 20 min. After 20 min, color changes were observed in the wells.

3.7 Anti-bacterial earphone buds coated with TBOSSNP

Commercially available silicone ear bud white colour was purchased from local shop in Coimbatore, India. The ear buds were cut into 0.5cm x 0.5cm size with a sterile scissor. The ear buds were put in distilled water and probe sonicated for 1h. Surface profiling of the ear buds before and after probe sonication was recorded using 3D optical Profilometre. The ear buds were then subjected to plasma treatment for 1h at plasma current 1.89A. TBOSSNP was coated on the ear buds by drop casting method, the ear buds were allowed to air dry, weight of the ear buds before and after coating was noted. One set of ear buds were coated with TBOSSNP and epoxy resin (1:1) by adopting the same pre-treatment procedures. All the ear buds were weighed before and after coating. Surface profiling of the TBOSSNP coated ear buds were also done. Anti-bacterial activity of the coated earbuds against *S.aureus* was carried out (**Appendix 2A**).

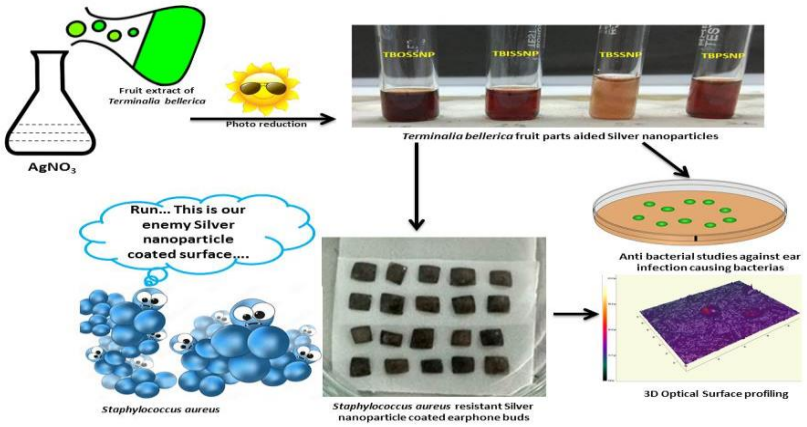


Figure 3. Schematic representation of fabrication and analysis steps involved in TBOSSNP coated antimicrobial earphone

The coated ear buds were subjected to leaching studies by dipping in water and wiping with water as real time cleaning process. The weight of the ear buds before and after leaching studies were noted. Triplicate studies were performed.

3.8 Bactericidal activity of GNP and SNP synthesized from cereals and pulses washed water

The anti-bacterial activity of synthesized nanoparticles BJAgSo, IBRRWM, IRaRWSoAg, BJWM, WCWM, WCAgSo and the washed waters (BJWW, IBRRW, IRaRW, and WCWW) were subjected to bacterial testing against common bacterial strains. Ciprofloxacin was chosen as standard. The detailed procedure is provided in **Appendix 2B**.

3.9 Anti-cancer activity of GNP and SNP from cereals and pulses washed water against A375 skin cancer cell lines

The anti-cancer activity of the nanoparticles depends on the shape, size and surface modification, this statement is obtained from the previous literature search. This study focus on the *in vitro* cell growth inhibition studies of the synthesized nanoparticles (BJAgSo, IBRRWM, IRaRWSoAg, BJWM, WCWM, WCAgSo) and the washed waters (BJWW, IBRRW, IRaRW, WCWW), Doxorubicin was taken as a standard against the A375 cell lines (Skin Malignant melanoma cells). The cytotoxicity of the nanoparticles was ascertained by MTT assay (**Appendix 3**).

3.9.1 Calculation of cell viability

The *in vitro* experiments were conducted three times, and statistical analysis was performed using SPSS version 17.0. It is important to note that a significant P value of 0.01 was considered in statistical analysis.

The following formula was used to calculate the cell viability,

$$\% \text{ viability} = \frac{\text{OD of Sample}}{\text{OD of Control}} \times 100$$

3.10 DNA binding studies of GNP and SNP

Interaction of synthesized nanoparticles with DNA was studied to understand the binding mechanism of metal nanoparticles to the DNA. The binding mechanism will actually help to correlate the interaction of the nano drugs to the human body. For this the synthesized nanoparticles BJAgSo, IBRRWM, IRaRWSoAg, BJWM, WCWM and WCAgSo was treated with the calf thymus DNA (ct-DNA) by adopting the previously reported procedure (**Firdhouse and Lalitha, 2015**).

3.10.1 Spectrophotometric analysis of DNA-nanoparticle interaction

The nanoparticles (BJAgSo, IBRRWM, IRaRWSoAg, BJWM, WCWM and WCAgSo) 10 µL each from the prepared concentrations (1 x 10^{-9} M, 3 x 10^{-9} M, 5 x 10^{-9} M, 7 x 10^{-9} M) were allowed to interact with 10 µL of ct-DNA (3 x 10^{-9} M) incubated at 24^{o}C. The OD @ 260/280 value was noted at four different time intervals (30min, 60min, 90min and 120min) in Biospec nano spectrophotometer (Shimadzu) with dsDNA mode with 4µL of nanoparticle-DNA complex solutions. The OD @ 260/280 of samples which are not incubated in the ct-DNA was also recorded for comparison.

3.11 *Allium cepa* root tip toxicity assay for Metallic nanoparticles

To determine the toxic effect of synthesized nano particles in chromosomal level, a simple toxicity assay was carried out with the meristamatic root tips of *Allium cepa* as per the previously reported methods (**Rajeshwari *et al*., 2016 and Mangalampalli *et al*., 2018**).

Allium cepa var.aggregatum commonly called as shallots are used for the study (purchased from local market in Coimbatore, Tamil Nadu, India). Healthy and fresh bulbs were placed in water by dipping root portion in water at room temperature. The water in the tube is replaced with fresh water daily. The root tips were incubated in BJWM (12.5µg/mL, 25µg/mL, 50µg/mL and 100µg/mL), distilled water and $K_2Cr_2O_7$ (100µg/mL) for 4h once the root elongates to 2-3cm. Triplicate studies for each tube is carried out for minimizing the error. The root tips from incubated roots were taken and fixed with Glacial acetic acid: Ethanol (1:3 v/v) solution. The tips were hydrolysis with 1M HCl for 5 minutes followed by staining with Acetocarmine dye (9:1 Acetocarmine: 1M HCl v/v) solution and placed on a microscopic slide and heated.

Figure 4. Growing *Allium cepa* root tips in fresh water

Figure 5: *Allium cepa* root tips incubated in different concentration of gold nanoparticles

The stained tip is bound with a drop of glycerol and covered with a cover slip. The root tip is squashed manually and microscopic analysis was carried out using a trinocular USB light microscope with 1000x magnification (Olympus CX 33) where the cytological changes of the incubated root tips were analyzed and the images of the sample were recorded with Magvision software.

3.12 Sun screen lotion formulation incorporating nanoparticles

Cosmetic applications of ZnONP and GNP were executed by preparing lotion formulations incorporating them.

3.12.1 Preparation of lotion formulation incorporating HN28Au

Topical skin care lotions were prepared by incorporating the synthesized HN28Au. The sunscreen lotions were prepared by the addition of aqueous phase to the oil phase with continuous stirring. Emulsifying wax (10 mg) and Bees wax (10 mg) constituting the oil phase was heated up to 70 ± 5^{o}C. Aqueous phase consisting of Glycerol (8 mL) and cetyl alcohol (5mg) was heated up to 80^{o}C and was added to the oil phase drop wise with continuous stirring. To this mixture TiO_2 and HN28Au (2mL) was then added and stirred for 4h until homogeneity. The stirring was done by placing the container in a water bath (double boiling method). The formulation by adding HN28 (2mL) was also prepared. The formulation without HN28 and HN28Au serves as blank.

3.12.2 Preparation of lotion formulation incorporating ZnONP

Skin care lotion incorporating synthesized ZnO nanoparticle from IRaRW involves aqueous phase, Glycerol (8 mL) and Cetyl alcohol (5mg), Stearic acid (10mg), IRaRW, *Cynodon dactylon leaf* (CND) extract (hexane and aqueous) heated up to 80^{o}C. Oil phase consist of Emulsifying wax (10 mg) and Bees wax (10 mg) heated up to 70 ± 5^{o}C continuous stirring was followed by the addition of TiO_2 and RaZn (4mg). The container was placed in the water bath and stirring continued for 4h. The formulation without IRaRW, RaZn and CND extract was taken as blank.

3.12.2.1 Preparation of aqueous extract of CND

About 5g CND leaf is mixed with 100mL H_2O and heated under direct flame for 20min. The solution is cooled and filtered. The obtained filtrate is aqueous extract of CND and incorporated in the lotion formulation.

3.12.2.2 Preparation of hexane extract of CND : About 3.7583g CND is added to round bottom flask and 130mL of hexane is added and refluxed for 1h at 40 ^{o}C. The solution is cooled down and filtered; filtrate obtained is added in lotion formulation.

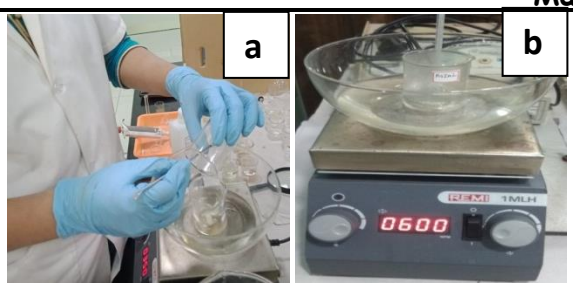


Figure 6: (a) addition of aqueous phase to oil phase, (b) mixture under stirring

3.12.2.3. Thin layer chromatographic separation of CND hexane extract: The prepared hexane extract is spotted in precoated silica gel plate (7cm x 3 cm). The mobile phase was fixed in trial and error basis starting from non-polar to polar solvents (petroleum ether, ethyl acetate, ethanol and formic acid).

3.12.3 Physico-chemical analysis of lotion formulations: The prepared topical skin lotion was then subjected to various analyses to determine its physiochemical properties. With reference to the IS 6608:2004 skin lotion specification, the prepared topical skin lotion undergone series of parameters such as pH, Thermal stability, Spreadability, *in-vitro* occlusivity and spectrophotometric determination of sun protection ability of the prepared skin lotion. Triplicate values were taken in each study.

3.12.3.1 Stability test: The prepared lotion samples were filled in the tube and incubated for 48h at 45^{o}C. The phase separation was recorded after the incubation period. This contributes to thermal stability. Another set of prepared formulations were taken in 1.5 mL centrifuge vials and centrifuged for 15 min in 3500 rpm. Visual separation of the composition if any was observed and noted.

3.12.3.2 Spreadability test: About 0.5g lotion was placed on a glass plate of 20 x 20 cm and covered with another plate of same dimension. Over this plate a 500g weight was kept for 5 minutes. The increase in diameter of the sample was noted.

3.12.3.4 Colour and pH test: The visual observation of the colour is noted. About 200mg lotion samples were dispersed in 20mL water. The pH of samples was analyzed in pre calibrated pH metre. The pH values were recorded

3.12.3.5 *In-vitro* occlusivity test: About 10mL water was filled in a 25mL beaker with diameter 3.2 cm and height 4.6 cm. The beaker was covered with a whatman filter paper (42) with 200 mg evenly distributed lotion sample and incubated for 48h at 37^{o}C. Beaker covered without any sample taken as negative control.

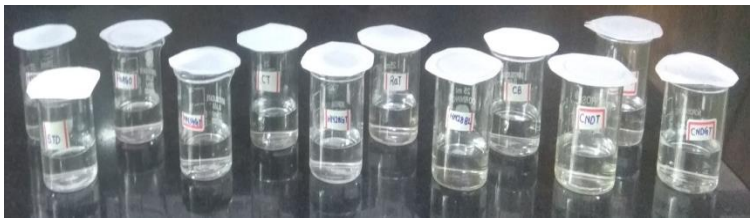


Figure 7. Samples prepared for occlusivity test before incubation

Occlusion factor F= A-B/ A * 100; A= Water reflux through uncovered filter paper; B= Water reflux through filter paper when covered by test preparation

3.12.4 Spectrophotometric determination of sun protection ability of lotion formulations

About 20mg lotion sample was dissolved in 1mL ethanol. The UV absorbance using Biospec Nano spectrophotometer was measured at different wavelengths 290nm, 295nm, 300nm, 305nm, 310nm, 315nm, 320nm. With the obtained absorbance sun protection factor was determined using the formula

$$^{320}_{290}\text{SPFSpectrophotometre} = CF*\sum EE\ (\lambda)*I\ (\lambda)*Abs\ (\lambda)$$

Where EE(I)-erythermal effect spectrum; I(i)-Solar intensity spectrum; Abs(I)-absorbance of sun screen product's; CF-correction factor (=10).

3.13 Nanoswitching/sensing application of nanoparticles

Nano switching application of nanoparticles when exposed to ammonia, cyanide and melamine was determined by colorimetric and electrochemical methods. The detailed procedure is as follows.

3.13.1 Colorimetric sensing of Ammonia by TBPGNP

The selective and sensitive colorimetric sensing potential of TB aided gold nanoparticle (TBPGNP) was determined. The synthesized gold nanoparticle was treated with equal volume of various chemicals and food adulterant solution, the change in colour is observed. The TBPGNP shows a visual colour change with only 1:1 ammonia solution. In order to assure the selective sensing of ammonia gas by the nanoparticle and not the ammonium ion, the TBPGNP solution was treated with the different ammonium salt solutions. The colour change of the solution if any was also recorded.

To determine the lowest concentration at which the visual detection of ammonia by TBPGNP is possible was tested. For this, different concentration of ammonia solution from 50,000ppm to 1ppm was prepared. To each concentration of the ammonia solution equal volume of TBPGNP was added. The colour change was recorded. UV-visible spectral analysis was carried out for the TBPGNP-ammonia solution. The spectra were compared with the TBPGNP without adding ammonia solution. The mechanism of action of TBPGNP for the detection of ammonia was determined from the spectral analysis.

3.13.2 Colorimetric sensing of cyanide by GCAu

Sensing behaviour of GCAu particles were visually detected by adding different chemical solutions to GCAu solution. The observed colour change is noted. To optimize the sensing behaviour of the GCAu particles, different concentration of cyanide $K_4[Fe(CN)_6].3H_2O$ solution were prepared (0.1M to 1M). The colour change was recorded.

3.13.3 Electrochemical cyanide sensing by GCAu@CPE

To analyze the sensitivity of the GCAu nanoparticle towards CN^- sensing, the nanoparticle incorporated carbon paste electrode was fabricated. The fabricated electrode was used to sense CN^- ions.

3.13.3.1 Fabrication of GCAu@CPE

GCAu @ CPE (GCAu1000) was fabricated by manual mixing of graphite powder and silicon oil (70:30 w/w) in a mortar. To this borax (100mg) was added and mixed. This mixture is considered as blank carbon paste. The carbon paste is modified with GCAu (1000μL) and mixing is continued until a homogenous paste is formed. The prepared modified carbon paste is then filled inside a Teflon tube (internal diameter 3mm) and a Cu rod was connected to the paste for electrical conductivity. The bottom of the tube was wiped a clean paper and smoothen the surface. This electrode was dried in hot air oven at 60^oC for 4h. Similarly GCAu300 electrode was fabricated following the same procedure by modifying the carbon paste with 300 μL GCAu.

3.13.3.2 Electrochemical cell setup and analysis

The electrochemical cell setup consists of electrolyte KCl (0.1M) with Phosphate buffer (pH 7.2) and $K_4[Fe(CN)_6].3H_2O$ (0.01M) with total volume 45ml was maintained at N_2 atmosphere. Three electrode system was setup with GCAu1000 (Working electrode-WE), Ag/AgCl (reference electrode) and Pt wire (counter/auxiliary electrode) dipped into the electrolyte solution and connected to the respective cable with alligator clips of electrochemical workstation. Cyclic voltammetric studies were carried out with potential ranges from -1V to +1V at room temperature. The peak currents were noted. The sample run without $K_4[Fe(CN)_6].3H_2O$ was considered as blank. The results were compared with the standard glassy carbon electrode. The studies were optimized by varying the scan rate and concentration of $K_4[Fe(CN)_6].3H_2O$. The studies are repeated with the GCAu300 electrode. Among the best electrode, sensitivity, selectivity and real sample analysis for sensing CN^- ions was carried out.

Figure 8. (a) Incorporating carbon paste into the teflon tube, (b) Electrochemical cell setup

3.13.4 Electrochemical cyanide sensing by low cost CPE in corn sticks

An economic fabrication of carbon paste electrode was done by filling the modified carbon paste into a corn stick. Corn sticks were collected from the local farm in Erode district, Tamil Nadu. The porous material inside the sticks was scrapped off and was cut to a length of 6.5cm. The Carbon paste was prepared by manual mixing of Graphite powder and silicon oil (70:30 w/w) in a mortar. To this borax (100mg) was added and mixed. This mixture is considered as blank carbon paste. This is modified by adding BJRGO (5mg), BuRGO (5mg) and TBPGNP (1mL) and mixed well. This modified carbon paste (CSC) is then filled inside the corn stick (internal diameter 3mm) and a Cu rod was connected to the paste for electrical conductivity. The bottom of the tube was wiped a clean paper and smoothen the surface. Conducting nature of the corn stick electrodes was determined by electrochemical studies.

The electrochemical cell set up consists of $K_4[Fe(CN)_6].3H_2O$ (0.01M) electrolyte with total volume 45mL was maintained at N_2 atmosphere. Three electrode systems was setup with CSC (Working electrode), Ag/AgCl (reference electrode) and Pt wire (counter/auxiliary electrode) dipped into the electrolyte solution and connected to the respective cable with alligator clips of electrochemical workstation. Cyclic voltammetric studies were carried out with potential ranges from -2V to +2V. The peak currents were noted.

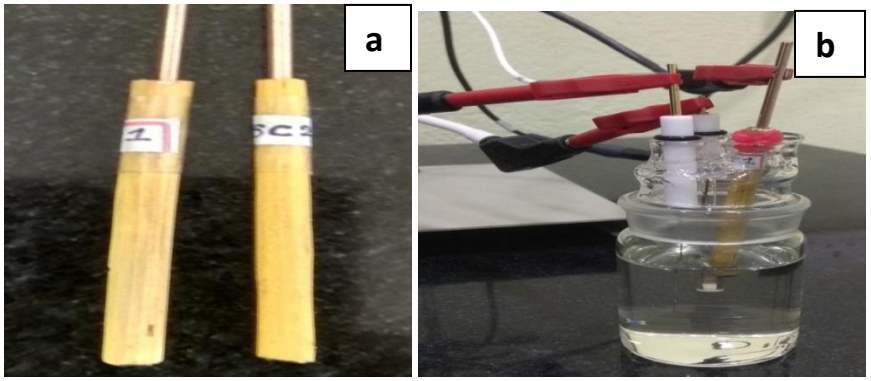


Figure 9. (a) Fabricated corn stick electrode (b) electrochemical cell set up with corn stick electrode

3.13.5 Analytical sensing of melamine leached out from Melamine tableware

Melamine leached out from melamine tableware when incubated in common Indian foods were determined by FTIR analysis. The detailed procedure is as follows.

3.13.5.1 Preparation of stock solution

Melamine (0.01M) was made by combining 0.5068g melamine with 300 mL of doubly distilled water. Throughout the course of the study, this common solution was utilized as a reference.

3.13.5.2 Melamine leaching studies

Melamine leaching investigations were conducted in accordance with standard procedures from European Union. The "melamine" wares utilized in the study were of two-year-old melamine plate and a "melamine" bowl (new). Hot foods (hot water, rice porridge, pepper rasam, and tomato soup) were separately put to the "melamine" wares and kept at 75°C (higher temperature) in a hot air oven for 2 h. Another experiment was carried out at 40°C (lower temperature). Another set of leaching studies was carried out in microwave oven. After cooling doubly distilled water in the refrigerator for 20 minutes, the cold water was transferred to a used melamine plate and heated in the microwave (300W, 70^{o}C). After 2.47 minutes, melamine dishware started to crack and make a popping noise coming from the microwave. The oven was shut off right away. The heating cycle was set to last for 5 minutes. The solutions were decanted into glass beakers after the heating phase, and an aliquot was collected for analysis. The food samples after incubation in melamine table wares are denoted as leached melamine samples in the following pages.

3.13.5.3 FT-IR analysis of leached melamine samples

The food samples were deposited on a glass plate (1cm x 1cm) and FT-IR analysis was done. The spectra of the samples are compared with standard "melamine" spectra". The food samples provided the background for each spectrum without incubation with melamine.

3.13.5.4 Comet assay determination of DNA damage by leached melamine samples

- **Sample preparation for comet assay**

We followed the procedure of **Firdhouse and Lalitha, 2015** to produce the stock ct-DNA. Highly polymerized ct-DNA fibers were sonicated and then stored in a 10 mL TRIS-HCl buffer (10 mM) solution at 4°C for 24 hours. We exposed food samples that had been in contact with melamine plates at 75°C to 10 μL ct-DNA at room temperature for 24 hours. Finally comet test was conducted on the samples to confirm the results (**Appendix 4**).

3.13.6 Electrochemical Melamine sensing by BJRGO@CPE

Melamine sensing by the carbon paste electrodes modified with synthesized reduced graphene oxide particles by electrochemical method was executed.

3.13.6.1 Fabrication of BJRGO@CPE

CPE was fabricated by manual mixing of graphite powder and silicon oil (70:30 w/w) in a mortar. To this borax (100mg) was added and mixed. This mixture is considered as blank carbon paste. The prepared modified carbon paste is then filled inside a teflon tube (internal diameter 3mm) and a Cu rod was connected to the paste for electrical conductivity. The bottom of the tube was wiped with a clean paper and smoothen the surface. This electrode (GSB) was dried in hot air oven at 60^{o}C for 4h.

Carbon paste electrode was modified with synthesized BJRGO (5mg) by mixing with the prepared carbon paste. This mixture was filled inside a teflon tube and connected

with a Cu rod for electrical conductivity. The bottom of the tube was wiped a clean paper and smoothen the surface. This electrode (GSBBJ(M)) was dried in hot air oven at 60^{o}C for 4h. The modification of carbon paste electrode was carried out by dipping the GSB electrode is BJRGO dispersed in H_2O for 24h. This modified electrode was designated as (GSBBJ (D)). GSBBJ (D) was dried in hot air oven at 60^{o}C for 4h.

3.13.6.2 Stability and reusability test for fabricated electrodes

The electrochemical cell setup for the study consists of electrolyte $K_4[Fe(CN)_6].3H_2O$ (0.01M) with total volume 45ml was maintained at N_2 atmosphere. Three electrode system was setup with Standard glassy carbon (GCE) (Working electrode), Ag/AgCl (reference electrode) and Pt wire (counter/auxiliary electrode) dipped into the electrolyte solution and connected to the respective cable with alligator clips of electrochemical workstation. Cyclic voltammetric studies were carried out with potential ranges from -1V to +1V. The peak currents were noted. The studies are repeated with the GSB electrode in the same electrolyte solution. The peak values were noted. To the same solution again GCE was inserted and study was repeated with same parameters. To this again the GSB electrode washed and dried electrode was connected and continued the procedure with same parameters. The peak current values were compared. The similar procedure was continued with GCE, GSBBJ (D) and GSBBJ (M).

Figure 10. Fabricated carbon paste electrode and Standard glassy carbon electrode

3.13.6.3 Electrochemical cell setup for melamine sensing

GSBBJ (5) electrode with 5mg BJRGO mixed with carbon paste electrode was used as working electrode for the sensing of melamine. The electrochemical cell set up consists of electrolyte KCl (0.1M) with Phosphate buffer (pH 7.2) and Melamine (100nM) with total volume 45mL was maintained at N_2 atmosphere. Three electrode systems was setup with GSBBJ (5) (working electrode), Ag/AgCl (reference electrode) and Pt wire (counter/auxiliary electrode) dipped into the electrolyte solution and connected to the respective cable with alligator clips of electrochemical workstation. Cyclic voltammetric studies were carried out with potential ranges from -1V to +1V. The peak currents were noted. The sample run without melamine was considered as blank. The study was optimized with various scan rates and concentration of melamine. With the optimized results the detection of melamine in real food products were carried out. The selectivity and sensitivity of the electrode were determined in the presence of simulated interfering agents. The peak current value obtained from the cyclic voltammogram was noted.