III. METHODOLOGY

The present study titled**"Synthesis of Eco-friendly Nanogranular Films in Food Packaging using Medicinal plants and Nanoparticles"**was carried out under the following headings :

PHASE I

SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL STUDY OF SILVER NANOPARTICLES

A. Synthesis and Optimization of Silver Nanoparticles

1. Selection of Medicinal Plants from Pilot Study
2. Collection and Authentication of Medicinal Plants from Botanical Survey of India (BSI)
3. Home Gardening of Medicinal Plants
4. Preparation of Plant Powder
5. Optimization of Silver Nanoparticles from *Leucas aspera*
6. Determination of Particle size and Zeta potential of Medicinal Plants
7. Determination of Antioxidant Activity of Synthesized Silver Nanoparticle (AgNPs)
8. Analysis for Antimicrobial Activity

B. Characterization of *Glycyrrhiza glabra* Silver Nanoparticles

1. Identification of *Glycyrrhiza glabra* Constituents
2. Identification of Individual Compounds
3. Determination of Functional Groups
4. Elemental Analysis
5. Morphological Studies of Synthesized *Glycyrrhiza glabra* Silver Nanoparticles
6. Determination of Structure and Crystallinity Studies
7. Determination of Anticancerous Activity

PHASE II

COATING OF SILVER NANOPARTICLES ONTO COMMERCIALLY AVAILABLE FOOD PACKAGES AGAINST*ENTEROPATHOGENIC SPECIES*

C. Assessment of Antimicrobial Property and Shelf life of Nanocoated Food Packages :

Specifications of Commercially Available Food Packages :

1. PET bottles

- Shelf-life of Tomato Puree
- Shelf-life of Lemon Juice

  Material – Poly Ethylene Terephthalate (PET)

  Neck size – 25 mm

  Weight – 12.5 gms

  Colour – Transparent White Colour with Cream Cap

  Shape – Round

2. Infant feeding bottles

- Shelf life of Milk and Lemon Juice

  Material – PP (food grade)

  Description – Available in 4 oz (150 ml) Sizes with Multi Colour Printing

  Accessories – PP Feeding Bottle, Hood Cap Set and Latex Nipple

3. Ziploc covers

- Shelf life of Tomato Puree in Ziploc Covers

  Material – Poly Propylene (PP)

  Package contents – 100 g

  Size – 7 cm X 11 cm; 8” X 4.3” (W XL)

  Thickness – 2 Mil

  Net weight – 33 g

  Colour – Clear, Transparent

PHASE III

SYNTHESIS, OPTIMIZATION AND CHARACTERIZATION OF NANOGRANULAR EDIBLE FILMS

D. Synthesis and Standardization of Silver Nanoparticles (*Glycyrrhiza glabra)* Edible Films

E. Characterization of Edible Films

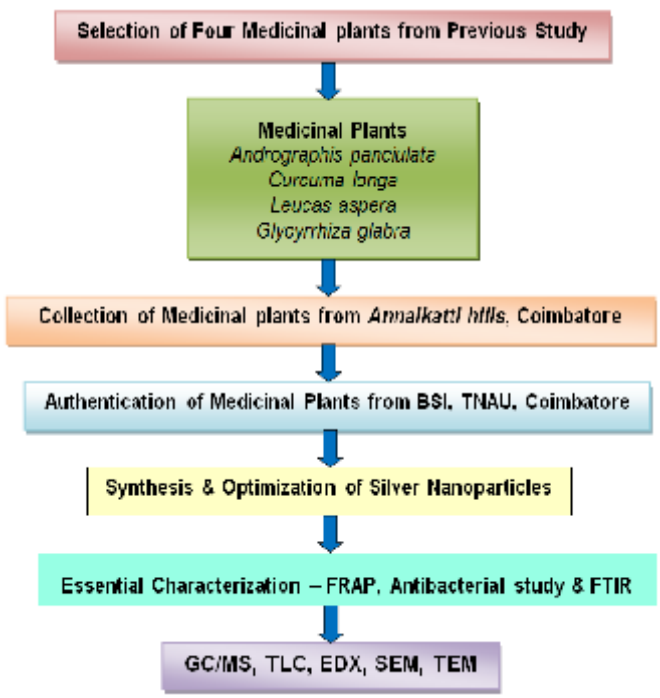
1. Thermal Analysis by Differential Scanning Calorimetry (DSC) (Q2000, USA)
2. Thermo Gravimetric Analysis (TGA, 2920, USA)
3. X-Ray Diffraction (XRD) (Rigaku Miniflex, Japan)
4. Water Vapor Permeability (WVP)
5. Antibacterial Testing of Edible films with Inoculated Bacterial Culture

The Research Design of the Methodology followed for the Study is given in Figure 1.

RESEARCH DESIGN

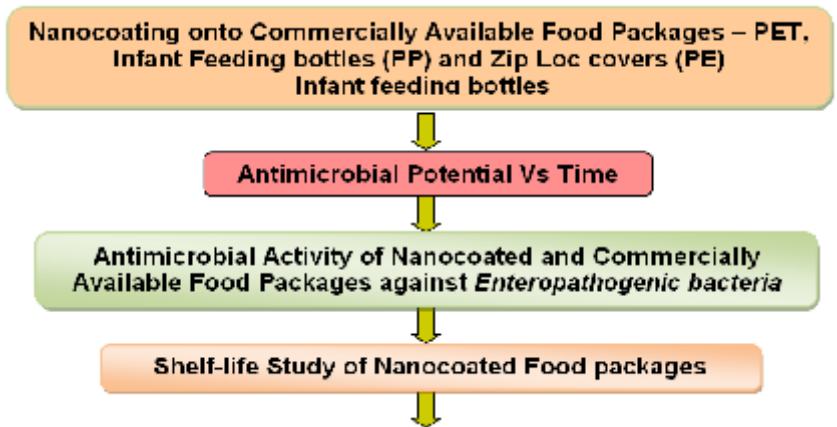
Phase I

Synthesis, Characterization and Antimicrobial Study of Silver Nanoparticles



Phase II

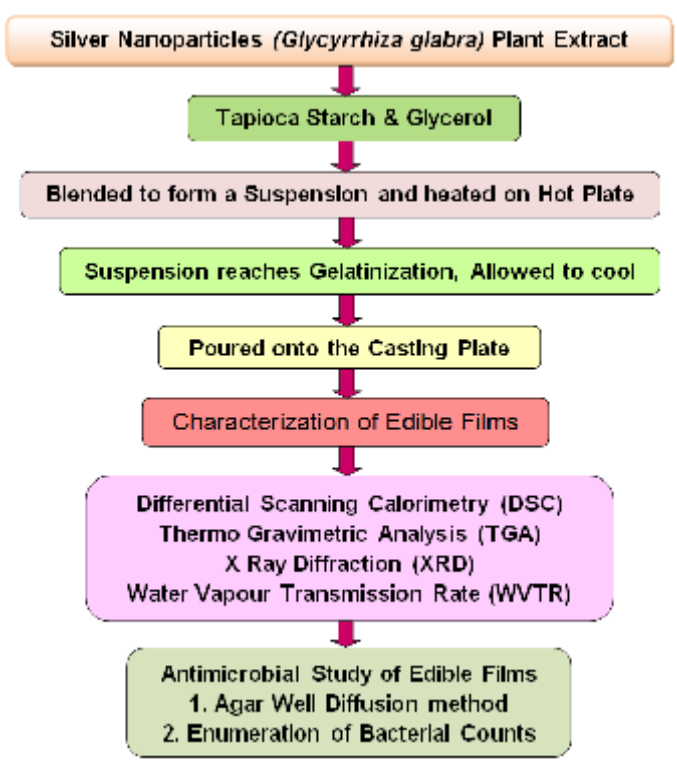
Coating of Silver Nanoparticles onto Commercially Available FoodPackages



Nanocoated food packages	Period of testing	Duration
PET bottles	Every 2 days	10 days
Zip Loc covers	Every 2 days	10 days
Infant Feeding Bottles	Every ½ an hour	3 hours

Phase III

Synthesis, Optimization and Characterization of NanogranularEdible Films



Phase I

Synthesis, Characterization and Antimicrobial Study of Silver Nanoparticles

A. Synthesis and Optimization of Silver Nanoparticles

1. Selection of Medicinal Plants from Pilot Study

A pilot study was carried out to study the mechanism of inhibition of microbes from twelve different plants namely *Azadirachta indica*, *Acorus calamus*, *Trigonella foenum-graecum*, *Piper longum*, *Foeniculum vulgarae*, *Andrographis paniculata*, *Brassica juncea*, *Glycyrrhiza glabra*, *Centella asiatica*, *Trachyspermum copticum, Curcuma longa* and *Leucas aspera*. Among these, four medicinal plants were found to exhibit higher antimicrobial property, based on their particle size, surface charges, stability, functional groups, morphology and phyto reduction of silver nanoparticles. These four medicinal plants namely *Andrographis paniculata, Curcuma longa, Leucas asperaand Glycyrrhiza glabra* were selected for the present study. Specific parts namely leaves, rhizome, leaves and root respectivelywere dried in the shade (Figure 2 – 5).

***Andrographis paniculata* (Leaves)**
Figure 2

***Curcuma longa*(Rhizome)**
Figure 3

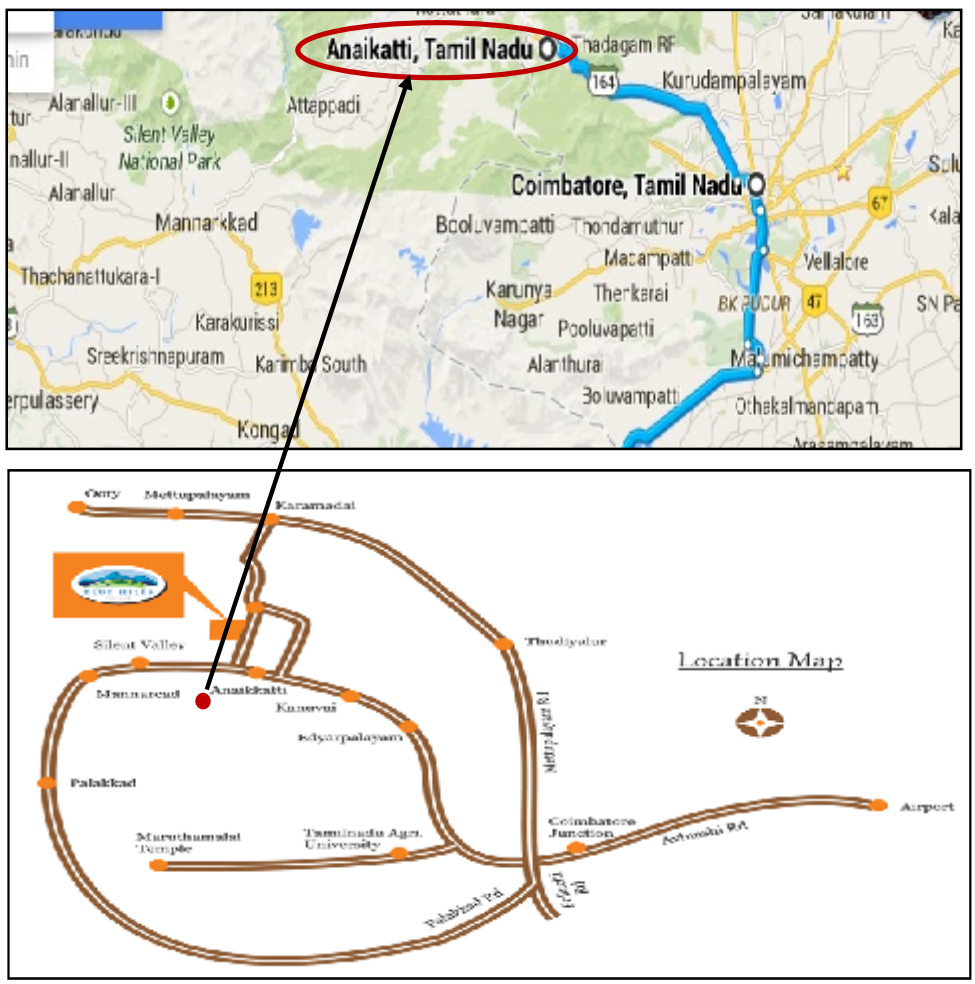
***Leucas aspera* (Leaves)**
Figure 4

G*lycyrrhiza glabra*(Root)
Figure 5

An attempt was made to identify the interaction between the nanocoated food packages and microbes in the prevention of *Enteropathogenic* disease.

2. Collection and Authentication of Medicinal Plants from Botanical Survey of India (BSI)

The Medicinal Plants were collected from Annaikatti hills, AIM (All India Movement) for Seva, Green Kovai, Anaikatti, Coimbatore, Tamil Nadu, India. Figure 6 denotes the geographical location of Green Kovai, Anaikatti Hills from Coimbatore District. These plants were identified and authenticated by Botanical Survey of India, under the Ministry of Environment and Forest, Southern Regional Centre, TNAU, Coimbatore, India.



Anaikatti Hills, Coimbatore District, Tamil nadu

Figure 6

3. Home Gardening of Medicinal Plants

It is evident from several literature studiesthat the home gardening of medicinal plants is widely used as a alternative remedy to alleviate illness and promote healthy lifestyle. Therefore, the four selected medicinal plants namely *Andrographis paniculata, Curcuma longa, Leucas aspera* and *Glycyrrhiza glabra* were grown in the home garden (Figure 7) using organic manure (Figure 8) Ayurvedha Vaidhya Centre, Coimbatore. Kitchen waste which is also an organic manure was used to increase the productivity of the home garden.

Potted Medicinal Plants

Figure 7

Organic Manure

Figure 8

4. Preparation of Medicinal Plant Powder

The fresh medicinal plants were collected from *Annaikatti* Hills, Coimbatore, Tamil Nadu, India. The specific parts namely leaves, root and rhizome were separated and shade dried (Figure 9) to completely remove dampness. They were then grated into small pieces and pulverized separately in a mixer and stored separately in air tight containers.

Shade Drying of Medicinal Plants

Figure 9

5. Optimization of Silver Nanoparticles (AgNPs) from *Leucas aspera*

For the production of extract, 10g of the plant powder was added to 100ml distilled water and then boiled for five minutes. The boiled extract was filtered using Whatmann filter paper No.1. Further, plant extract was added to 1mM $AgNO_3$ and centrifuged to obtain silver nanoparticles. The appearance of yellowish–brown color in aqueous solution indicates the onset of reaction which slowly turns into dark brown color at the completion of reduction of silver Ag^{+} to Ag^{0}.

a. Trial 1

Among four medicinal plants, *Leucas aspera*was selected to synthesis and standardize the silver nanoparticles. Added 10g of the plant powder to 100 ml distilled water and then boiled for five minutes. The boiled extract was filtered using Whatmann filter paper No.1. Further 1ml of the supernatant was added to 9ml of 1mM $AgNO_3$ and then centrifuged through General Laboratory Centrifuge (GLC) with (g value of 2000) for 10 minutes to obtain silver nanoparticles.

b. Trial 2

Trial 2 was carried out by following the trial 1 method with General Laboratory Centrifugation (GLC) (g value of 2000) for 20 minutes and the supernatant was collected to obtain silver nanoparticles.

c. Trial 3

Trial 3 was conducted by following trial 2 method with General Laboratory Centrifugation (GLC) (g value of 2000)for 30 minutes to yield silver nanoparticles.

d. Trial 4

Trial 4 was carried out by General Laboratory centrifugation (GLC)(g value of 2000) for 45 minutes (g value of 2000) to obtain silver nanoparticles.

e.Trial 5

Trial 5 was repeated centrifuge for 15 minutes with ultracentrifugation (g value of 12,250), instead of GLC to obtain smaller nanoparticles <100 nm.

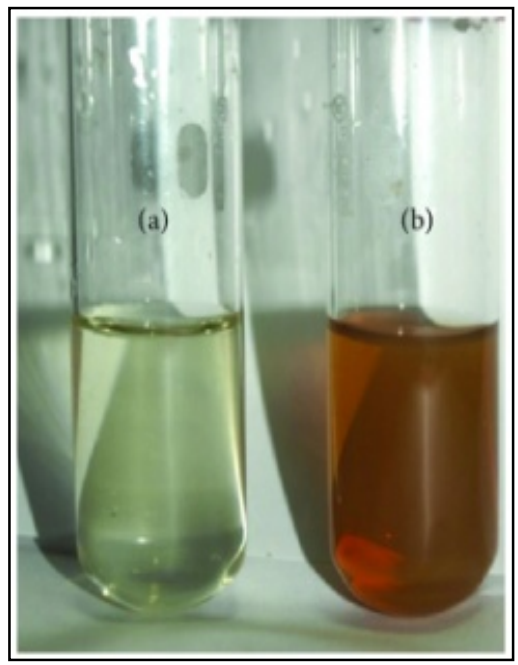
f. Trial 6

Similarly, trial 6 was carried out ultracentrifugation (g value of 12,250) for 45 minutes to yield nanoparticles.

g. Trial 7

Similarly, Trial 7 was ultra centrifuged (g value of 12,250) for 1 hour 10 minutes to yield silver nanoparticles, based on the previous preliminary studies (Adaline *et al.,* 2011).

Among twelve different medicinal plants, only four plants namely *Leucas aspera, Andrographis paniculata, Glycyrrhiza glabra and Curcuma longa* showed better antimicrobial activity. Hence, these plants were selected for the present study. To start with *Leucas aspera* was used for the synthesis and optimization of silver nanoparticles,through general laboratory centrifugation (GLC) and ultracentrifugation to achieve smaller particle size. When the plant extract was added to 1mM $AgNO_3$ (1: 9), silver nanoparticles were formed with a colour change from light yellowish to dark brown, indicating the reduction of silver nanoparticle, due to phytochemicals in the aqueous solution (Figure 10). However, the intensity of colour change of the nanoparticle solution depends on the medicinal plants.



Colour Change by Bio-reduction of Medicinal Plant
(a - Medicinal plant extract b-Nanoparticle solution)

Figure 10

a. Effect of Processing Method on Nanoparticle Suspension

(i) General Laboratory Centrifugation (GLC)

Particle size and zeta potential were analyzed simultaneously to assess both the size distribution and corresponding suspension stability. The particle size distribution is observed by Diffuse Light Scattering (DLS) method (Nano ZS 90, Malvern Instruments Ltd, UK). The particle size analysis reflects the retention of unfoliated Ag particle and also the activity towards agglomeration. The desired size of the nanoparticle could be achieved by controlling concentration of plant extracts, reaction time and temperature. Generally, zeta potential values are taken as indicative criteria to estimate the longevity of suspension. Zeta potential value reveals the surface charge and stability of the synthesized silver nanoparticles. The zeta potential analysis indicates that the sustainment of silver nanoparticles and its activity under steady state condition. Table I presents the details on Optimization of Silver Nanoparticles of *Leucas aspera* based on particle size and zeta potential.

TABLE I

OPTIMIZATION OF SILVER NANOPARTICLES *(Leucas aspera)* BASED ON PARTICLE SIZE AND ZETA POTENTIAL

Centrifugation time (minutes)	Average Size (nm) (a)	Zeta Potential (mV) (b)	Figure No.
GLC - 10	167.90	-29.6	11
GLC - 20	148.10	-29.2	12
GLC - 30	115.10	-18.4	13
GLC - 60	63.45	-18.1	14
Ultra centrifuge - 15 mins	60.40	-30.5	15
Ultra centrifuge - 45 mins	51.10	-26.0	16
Ultra centrifuge - 1hr 10 mins	23.44	-24.70	17

*GLC – General Laboratory Centrifuge

Among the four medicinal plants chosen to start with, *Leucas aspera*AgNP was synthesized and optimized by using general laboratory centrifugation for 10 minutes (GLC- 10 minutes) with a g value of 3000. There were two distributions of size ranging from 30 nm – 200 nm, 100 nm – 500 nm possessing an average particle size of 167.90 nm (Figure11 A) between 50 nm and 300 nm respectively. The corresponding suspension stability of Z potential were 29.6 mV and 20 mV (Figure 11 B) respectively.

Further, the same *Leucas aspera* nanoparticle suspension was subjected to prolonged time, by increasing the time of centrifugation to 20 minutes (GLC – 20 minutes) with a g value of 3000. Three distributions were achieved ranging from 25 nm – 225 nm, 375nm – 1150 nm, 4500 nm – 6000 nm achieving an average particle size of 148.10 nm (Figure 12 A) between particle size of 70 nm – 5000 nm respectively. The same solution corresponded to the suspension stability of -29.2 mV and 20 mV respectively, (Figure 12 B), when centrifuged for 20 minutes.

To obtain smaller particle size, the same suspension of *Leucas aspera* nanoparticle was further subjected to GLC by increasing the centrifugation time to 30 minutes (GLC - 30 minutes) with a g value of 3000. Therefore, the peak distribution range from 20 – 600 nm possessing an average particle size of 115.10 nm (Figure 13 A). The corresponding suspension stability by zeta potential of -18.4 mV and 10 mV (Figure 13 B) respectively was observed.

When the same colloidal suspension of *Leucas aspera* was subjected by increasing the time of centrifugation to 60 minutes (GLC - 60 minutes) with a g value of 3000, there were two distributions namely 6 – 20 nm and 25 – 350 nm possessing an average particle size of 63.45 nm (Figure 14 A) between 10 – 100 nm range. The corresponding suspension stability were -18.1 mV and 10 mV respectively (Figure 14 B). However, results of the above optimization process of *Leucas aspera* nanoparticle suspension through GLC (10 – 60 minutes) revealed that a minimum particle size of 63.45 nm with a suspension stability of -18.1 mV respectively could be achieved. It was observed that over the increase of centrifugation time nanoparticle suspension with smaller particle size could be produced. In order to obtain smaller particle size < 50 nm and highly stable suspension, ultra centrifugation with a higher g value (12,000 g) is used as an alternative to GLC in the optimization process of *Leucas aspera* nanoparticle.

On ultracentrifugation for 15 minutes with 12,000 g a peak distributionwas observed with an average particle size of 51.1 nm (Figure 15 A) between 35 nm– 1225 nm and the corresponding suspension stabilities of -29.6 mV and 20 mV respectivley (Figure 15 B). Further, the same suspension was subjected to ultra centrifugation for 45 minutes (g value of 12,000). Two distributions were oberved ranging from 3.5 nm – 10 nm, 10 – 700 nm possessing an average particle size of 50 nm (Figure 16 A). The corresponding suspension stability possessed -24.7 mV and -20 mV zeta potential respectivley (Figure 16 B). As observed, smaller silver nanoparticles were obtained by increasing the time of centrifugation. Therefore, ultra centrifugation was carried out to 1 hr 10 minutes (g value of 12,000) producing a sharper and narrower distribution of particles with an

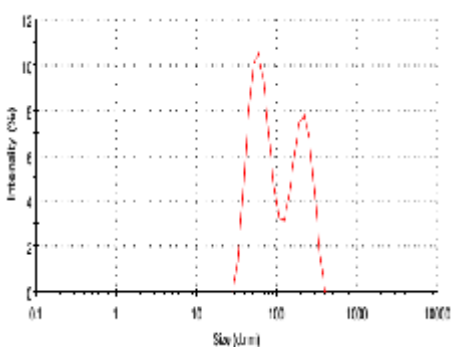
average particle size of 23.44 nm (Figure 17 A) ranging from 20 nm to 30 nm and the corresponding suspension stability are -24.7 mV and 20 mV respectively (Figure 17 B).

Hence, from all the above optimization methods of *Leucas aspera* nanoparticle, it was observed that ultracentrifugation (1 hr 10 minutes) of *Leucas aspera,* produced smaller particle size with a highly stable solution. Hence, ultra centrifugation (1 hr 10 minutes) with a g value (12,000) was adopted as the optimized method for synthesizing the Ag nanoparticles for the other medicinal plant extracts.

Figures 11 to 14 depict the particle size and zeta potential for different durations of GLC and Figures 15 to 17 shows the different durations of ultracentrifugation.

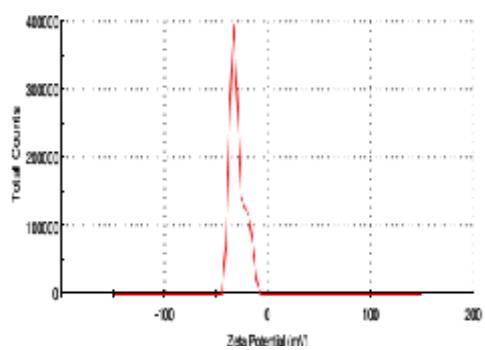
General Laboratory Centrifuge (GLC) – *Leucas Aspera*

Particle Size

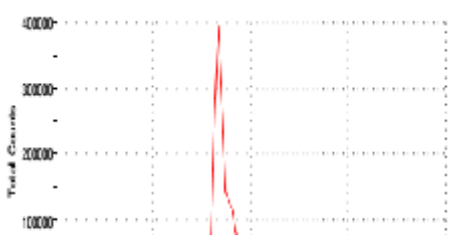


GLC - 10 Minutes
Figure 11 A

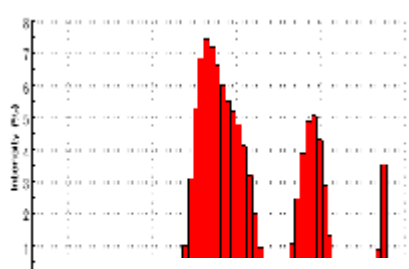
Zeta Potential



GLC - 10 Minutes
Figure 11 A



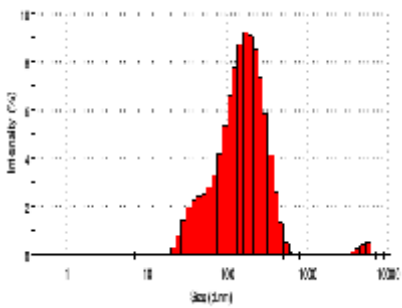
GLC - 20 Minutes
Figure 12 A



GLC - 20 Minutes
Figure 12 B

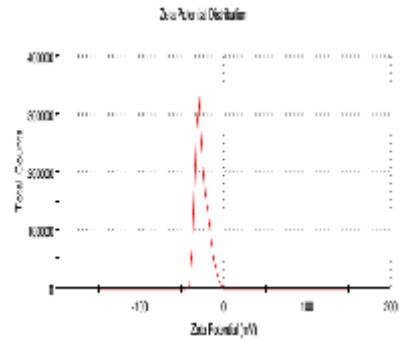
General Laboratory Centrifuge (GLC) – *Leucas Aspera*

Particle Size

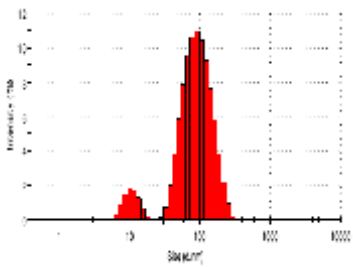


GLC - 30 Minutes
Figure 13 A

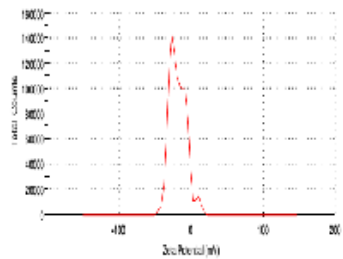
Zeta Potential



GLC - 30 Minutes
Figure 13 B



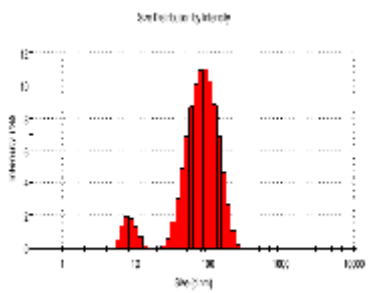
GLC - 60 Minutes
Figure 14 A



GLC - 60 Minutes
Figure 14 B

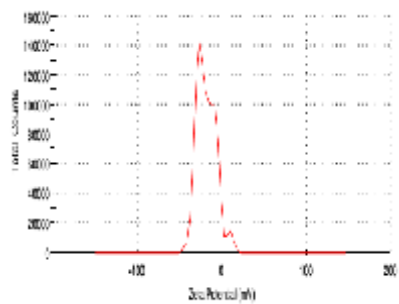
Ultracentrifuge – *Leucas Aspera*

Particle Size

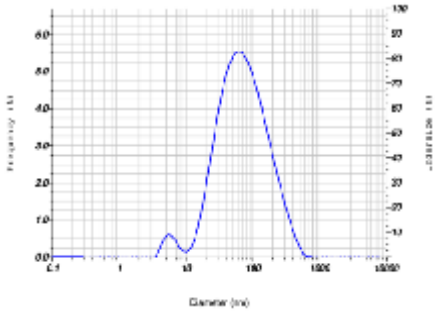


Ultra Centrifuge - 15 Minutes
Figure 15 A

Zeta Potential

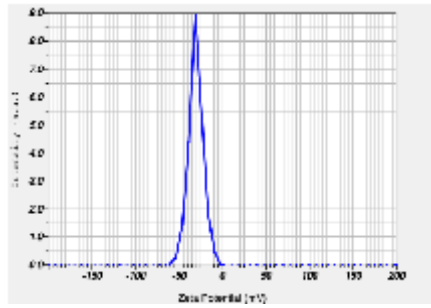


Ultra Centrifuge- 15 Minutes
Figure 15 B



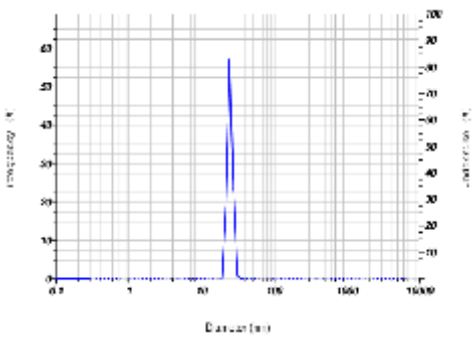
Ultra Centrifuge- 45 Minutes

Figure 16 A



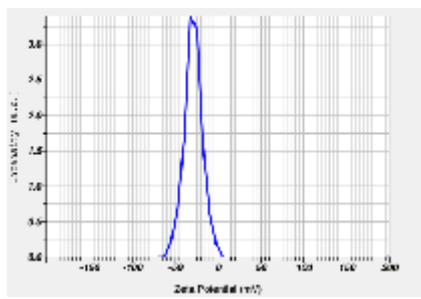
Ultra Centrifuge - 45 Minutes

Figure 16 B



Ultra Centrifuge -1 Hr 10 Minutes

Figure 17 A



Ultra Centrifuge - 1 Hr 10 Minutes

Figure 17 B

ii. Ultra Centrifugation

The ultra centrifugation (1hr 10 minutes) method with g value of 12,000 was adopted as an optimized method for synthesizing silver nanoparticles from the other three medicinal plants namely *Andrographis paniculata, Curucuma longa* and *Glycyrrhiza glabra.* Table I presents the Particle size and zeta potential of Silver Nanoparticles from other Medicinal Plants.

TABLE II

PARTICLE SIZE AND ZETA POTENTIAL OF SILVER NANOPARTICLES FROM OTHER MEDICINAL PLANTS (ULTRA CENTRIFUGATION FOR 1 HR 10 MIN)

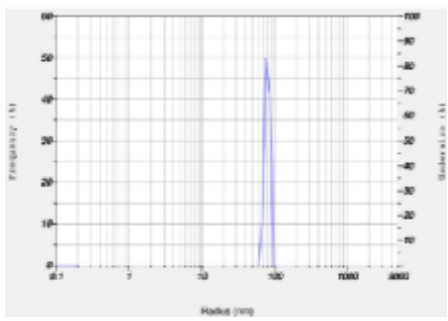
Medicinal Plants	Average Size (nm) (a)	Zeta Potential (mV) (b)	Figure No
Leucas aspera	23.44	-24.7	17
Andrographis paniculata	74	-20.0	18
Curucuma longa	127.4	-10.2	19
Glycyrrhiza glabra	34.7	-28.9	20

Silver nanoparticles synthesized from *Andrographis paniculata*, using the optimized standardization (ultra centrifugation 1 hr 10 minutes) showed distribution size ranging from 60 nm to 100 nm possessing an average particle size of 74 nm (Figure 18 A). The corresponding suspension stability (Zeta Potential) are -20 .0 mV respectively (Figure 18 B). Similarly, the above optimized methods were used for the synthesis of silver nanoparticles from *Curucuma longa*that resulted in a distributionfrom100 nm to 150 nm with an average particle size of 127.4 nm (Figure 19 A). The corresponding suspension stability of *Curcuma longa* nanoparticles were - 10.2 mV and 10 mV respectively (Figure 19 B).

Among all the nanoparticles synthesized from the four medicinal plant extracts, it has been observed that *Glycyrrhiza glabra* AgNP showed a narrow and sharper size distribution ranging from 25 nm to 45 nm possessing an average particle size of 34.7 nm (Figure 20 A) and the corresponding suspension stability as - 28.9 mV (Figure 20 B). The zeta potential of *Glycyrrhiza glabra*

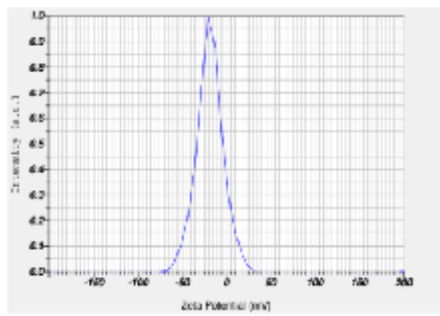
showed the highest stability when compared to other synthesized silver suspension of plant extracts. This study indicates the difference in sustainment of silver nanoparticles in suspension and its activity under steady state condition. The zeta deviation of the freshly prepared of *Glycyrrhiza glabra* is highly active resulting in chaotic motion observed through severe deviation of zeta potential. The conductivity of the particles was measured irrespective of the laser light scattering method.

Ultracentrifuge - *Andrographis Paniculata*



Particle Size

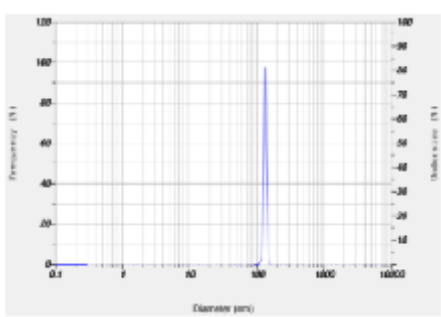
Figure 18 A



Zeta Potential

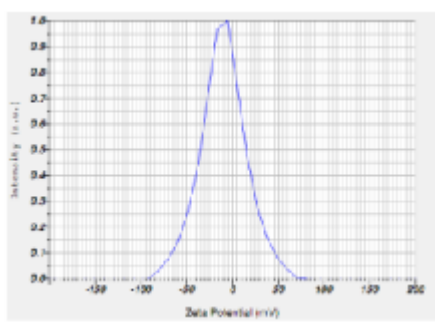
Figure 18 B

Ultracentrifuge - *Curcuma Longa*



Particle Size

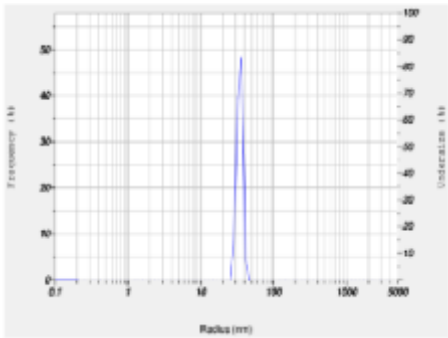
Figure 19 A



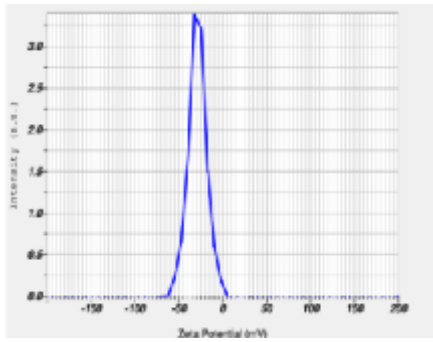
Particle Size

Figure 19 B

Ultracentrifuge - *Glycyrrhiza Glabra*



Particle Size
Figure 20 A



Zeta Potential
Figure 20 B

6. Determination of Particle size and Zeta Potential of Medicinal Plants

The Mean Particle Size and Zeta potential of the Silver Nanoparticles were determined by dynamic light scattering using a zeta size analyzer (Nano ZS 90, Malvern Instruments Ltd., UK). The stability and surface charge of synthesized silver nanoparticles were analyzed by using Malvern Zetasizer ZS 90. The samples were diluted 10 fold using 0.15 M PBS (pH 7.4) at a refractive index of 1.52 for better analysis.

7. Determination of Antioxidant Activity of Synthesized Silver Nanoparticles (AgNPs) (by Ferric Reducing Ability of Plasma as a Measure of Antioxidant Power - FRAP)

FRAP assay was performed according to the methods of Benzie and Strain (1999). A known volume of the sample was made up to 3 ml with phosphate buffer, 1 per cent potassium ferri cyanide and incubated in water bath for 20 minutes at 50°C, cooled and added to the 10 per cent Trichloroacetic acid, 2.5 ml distilled water and 0.5 ml Ferric Chloride, kept for 10 minutes at room temperature and the absorbance at 700 nm was read against standard ascorbic acid equivalent.

Calculation:

$$\frac{\text{Sample Absorbance}}{\text{Standard Absorbance}} \times \text{Standard Concentration}$$

8. Antimicrobial Study

The test microbes included in this study were enteropathogenic bacteria such as *Esherichia coli (MTCC 40), Salmonella enterica (MTCC3219)* and *Shigella dysenteriae (PSGIMS&R).* These cultures were obtained from MTCC, Chandigarh and PSG Institute of Medical Science and Research (PSGIMS&R), Coimbatore, Tamil Nadu, India. The pure cultures of bacteria were maintained on nutrient agar slants and maintained in refrigerator for use.

a. Enumeration of Bacterial Counts

The food packages namely PET containers, Zip Loc covers and Infant Feeding Bottles were selected for coating with AgNPs (nanocoated) which were compared and tested against standard packages (uncoated). The food stored in these food packages were periodically tested for every two days interval for a period of ten days. The nutrient agar plates were prepared aseptically and the foods were spread uniformly onto the plate by spread plate technique. These plates were incubated at 37^{o} C for 24 hours. The bacterial counts were made by Bacterial Colony Counters.

b. Assessment of Antimicrobial Activity

The objective of this experiment was to analyze antimicrobial potential of biosynthesized silver nanoparticle against the test organisms. The food packages were punched out into disc size for performing agar well diffusion method for determining the highest activity among the different concentration of nanoparticles. The antimicrobial activity of synthesized silver nanoparticles was determined by agar diffusion method. The log phase cultures were spread over the Nutrient agar medium plates using a sterile cotton swab in order to get a uniform microbial growth on test plates. Then approximately four wells of uniform sizes (0.65cm) were made with a cork-borer in two petrids. The petrid was loaded with 10µl, 20µl, 30µl, 40µl of silver solution and as other petrid was loaded with 50µl, 75µl, 100µl and 125µl of the encapsulated silver colloids. The silver colloids were tested against the organisms of *E.coli* (MTCC-40), *S.enterica* (MTCC 3219) and *Sh.dysenteriae (PSGIMS&R).* These plates were incubated at 37°C for 24-48 hours.

B. Characterization of *Glycyrrhiza glabra* Silver Nanoparticles

The characterization of Silver Nanoparticles was carried out by using the following techniques:

1. Identification of *Glycyrrhiza glabra* Constituents (using Gas Chromatography - GC)

The GC analysis was performed using a SHIMADZU QP 5050A, GC/MS-5989Binstrument. The GC was fitted with a DBI (30 m × 0.53 mm × 1.5 µm) fused with silica capillary column. The GC carrier was helium (flow rate 1ml/min) at an Ionization mode of EL 70eV. Temperature program was 40°C (static for 2 min), then increased to 160°Cat a rate of 2°C/min. The injection volume was 1µl. All the samples were analyzed in duplicate and the mass spectrum of each peak was analyzed to determine the mass to charge ratio, molecular weight and structure of each major compound in the samples.

2. Identification of Compounds (by Thin Layer Chromatography - TLC)

To determine the active compounds in the plant extract, using Thin Layer Chromatography, AgNPs were spotted on TLC sheet coated with thin layer of Silica gel (Macherey-Nagel, Germany) and the sheet was kept inside a chamber containing solvent (N- butanol, acetic acid and distilled water – 8 : 2 : 2 v/v) and allowed to develop the chromatogram. The TLC sheet was removed from the chamber and allowed to dry. After 20 minutes, 0.3% Ninhydrin was sprayed as developer and dried at 50- 60°C for 15 minutes. The activated TLC sheet was observed to elucidate the presence of active compounds from the plant extract.

3. Determination of Functional Groups (by Fourier Transform Infra-Red Spectroscopy - FTIR)

Infra-red spectroscopy was used to examine the physico-chemical interactions among different components in a nanoparticle formulation by using FTIR. The Silver Nanoparticles prepared after 48 hrs of reaction of the solution with plant powder broth, which was centrifuged at 10,000 rpm for 15 minutes, following which the pellet was redispersed in distilled water to get rid of uncoordinated biological molecules. The centrifugation process and redispersion

process was repeated three times to ensure better separation of free entities from the metal nanoparticles. The purified pellets were then dried. The powders were subjected to FTIR spectroscopy measurements. These measurements were carried out using a FTIR SHIMADZU 8400 S instrument with a wavelength range of 400 to 4000 nm. The samples were incorporated with KBr pellets to attain the spectra. The results were compared for shift in finger prints of functional groups.

4. Elemental Analysis (using Energy Dispersive Auger X ray analysis - EDX)

The silver nanoaparticles of dried and powdered form were subjected for EDX (Energy Dispersive Auger X –Ray) analysis for the determination of elements present along with the synthesized silver nanoparticles. The instrument used was EDX-OXFORD –INCA.

5. Morphological Studies of Synthesized *Glycyrrhiza glabra* Silver Nanoaparticles (using Scanning Electron Microscopy - SEM)

Morphological evaluation of the synthesized Silver Nanoparticles was carried out using a Hitachi S-4700 microscope (Hitachi High-Tech Corp. Tokyo, Japan). The samples were deposited onto an aluminum specimen stubs using double-stick carbon tabs (Ted Pella Inc., Redding, California., USA) and coated with gold/platinum using ion sputtering (Denton Vacuum Inc., Moorestown, N.J., USA) for 45 s at 20 mA. All samples were examined using an accelerating beam at a voltage of 1.5 KV magnifications of 20,000 X were used.

6. Determination of Structural and Crystallinity Studies (by Transmission Electron Microscopy - TEM)

Transmission Electron Microscopy (TEM, 200 kV, JEOL- 2010) was used to determine the shape, size and internal morphology of the synthesized nanoparticles. A couple of drops of Ag-nanoparticle suspension (concentration 50 µg/ml) was placed on carbon coated copper grid and dried inside a vacuum dryer. The copper grid was then scanned by TEM to examine the nanoparticles.

7. Determination of Anticancerous Property (by MTT Assay and Gel Electrophoresis)

HeLaand Liver carcinoma cell lines were seeded into a 96-well plate and incubated 24 hrs overnight at 37 °C to ensure cell adhesion and confluence in the wells. The medium was replaced with a fresh one containing silver nanoparticles and extracts from the plant in different concentrations (20–200 µl) for 24 hr. The effects of silver nanoparticles on the cells viability were estimated by MTT assay using 3-4,5-dimethylthiazol-2yl), 2,5-diphenyl-2H-tetrazolium bromide (MTT). For this, 10 µL of MTT solution was added to each well and the plates incubated for two hours in dark conditions, then 100µL DMSO added to solubilize the MTT. In the last part, the absorbance of each well was measured at 570 nm with a microplate spectrophotometer. Concentrations of silver nanoparticle in cell viability were then tested for their effectiveness in treating the cells and then compared with untreated cells (as control).

DNA fragmentation has long been used to distinguish apoptosis from necrosis, as the most reliable method for detection of apoptotic cells. HeLaand Liver carcinoma cells were plated in 16 six-well tissue culture plates and incubated at 37 °C overnight. These cells were treated with 2.0 ml of stock solution suspended in Dulbecco's modified eagle's medium (DMEM) containing 10% Fetal Bovine Serum. The cells were then, treated with 2 µg/mL Doxorubicin hydrochloride used as controls, along with the silver nanoparticle and extracts. The cell pellets were treated for 10 s with 50 µl of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min at 1600×g (unit rotations per minute) the supernatant was collected and the extraction repeated with the same amount of lysis buffer. With the supernatant, an additional amount of Sodium Dodecyl Sulphate (SDS) was added to a final concentration of 1% and treated for 2 hours with RNase A (final concentration 5 µg/µL) at 56 °C followed by digestion with proteinase K (final concentration 2.5 µg/µL) for 2 hours at 37 °C. By the addition of 0.5 µL 10 M ammonium acetate, DNA resulted in precipitated form with 2.5 µL ethanol for obtaining the DNA in a purified form. The DNA pellet was fused in 50 µL of TE buffer, and

separated by electrophoresis in 1.0% agarose gel containing 1 µg /mL ethidium bromide at 70 V. The DNA segments were visualized by exposing the gel to ultraviolet light, along with photography.

Phase II

Coating of Silver Nanoparticles onto the Commercially Available Food Packages against *Enteropathogenic species*

C. Assessment of Antimicrobial Property and Shelf-life of Nanocoated FoodPackages

The Food packages such as PET Bottles, Infant Feeding Bottles and Zip loc covers, were coated with highest concentration activity of AgNPs and compared with the standard packages (nonnanocoated), which were packed with acidic foods such as tomato puree and alkali foods, such as milk. It was inoculated with test organisms such as *E.coli* (MTCC 40), *Salmonella enterica* (MTCC 3219) and *Shigella dysenteriae (PSGIMS&R)*. Further, these foods were tested for a two days interval over a storage period of ten days, to check out the efficacy of nanocoated food packages on these foods on storage.

Phase III

Synthesis, Optimization and Characterization of Nanogranular Edible Films

D. Synthesis and Optimization of Nanoparticles (*Glycyrrhiza glabra)* Edible Films

Irradiated Cassava starch was obtained from Expande Company. Liquid Glycerol from Fischer Chemical Company. Silver nitrate (Batch No: 1089) from Sigma Aldrich (St.Louis, MO, USA) was used as received. Care was taken during handling regarding the photochemical reactions.

Teflon Casting Plates

Figure 21

The Teflon plates of ten inch diametre were used for synthesizing the edible films (Figure 21).

a. Trial 1

To initiate the study, 5 gmsof tapioca starch was suspended in 100 ml of water. To this was added 1.8% glycerol. With continuous stirring, 1% *Glycyrrhiza glabra* was dissolved in the remaining water and added to the starch suspension. This was kept dispersed on a hot plate at 70ºC for 15 minutes until gelatinization occurred. The films were made by casting the solution (100g) into ten inch diameter of teflon plates. These plates were oven dried at 45 ± 2 ºC for 12 hours, then the dried film peeled off from the surface of the plate (Figure 22).

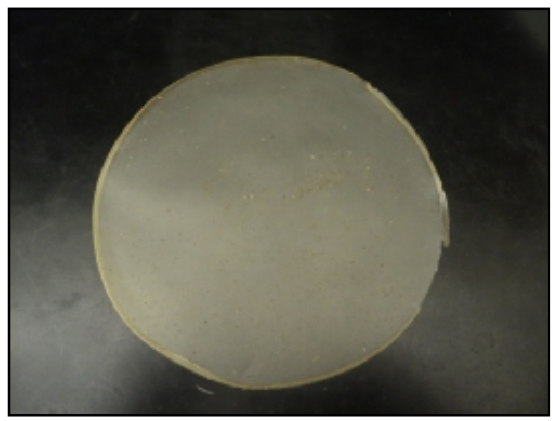
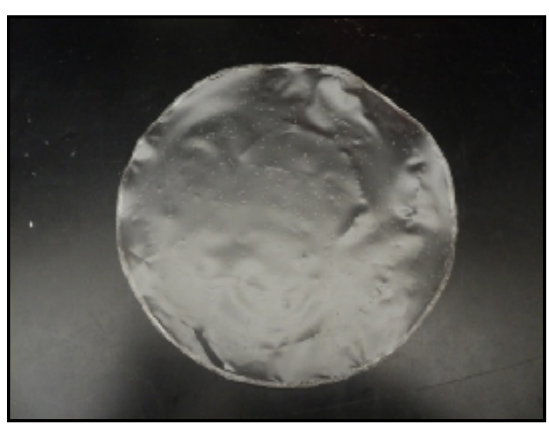


Figure 22

Tapioca Starch and Plant Extract

b. Trial 2

The edible films were prepared by dissolving 5 gmsof tapioca starch in 100 ml of distilled water, then added 0.7% acetic acid and 1.8% glycerol with constant stirring until it dissolves. To this, added 30 µl *Glycyrrhiza glabra* silver nanoparticle with constant stirring, dispersing the solution in hot plate at 70ºC for 15 minutes until gelatinization. These films were made by casting the solution (100g) into a ten inch diameter of teflon plates. These



Tapioca Starch, Plant Extract and Acetic Acid

Figure 23

plates were oven dried at 45 ± 2 ºC for 12 hours, then the dried film were peeled off from the surface of the plate (Figure 23).

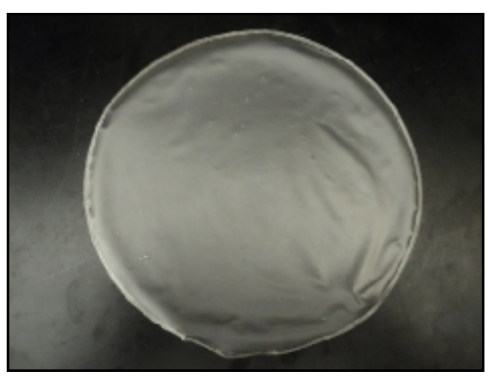
c. Trial 3

Similarly, trial 3 was carried out by following trial 2 method without adding 0.7 % acetic acid. 30 µl *Glycyrrhiza glabra* silver nanoparticle dispersed in water was added to the starch suspension. On further heating starch underwent gelatinization These films were made by casting the solution (100g) into a ten inch diameter of teflon plates. These plates were oven dried at 45 ± 2 ºC for 12 hours, then the dried film were peeled off from the surface of the plate (Figure 24). The percentage composition of the edible film before and after drying are given in Table III

TABLE III

PERCENTAGE COMPOSITION OF EDIBLE FILMS
(30 µl of Nanoparticle Impregnated Edible film)

Weight (%)		
Composition	**Before drying**	**After drying**
Tapioca Starch	5	73.52
Glycerol	1.8	26.47
Plant extract	0.0003	0.0044
Silver (30 µl)	0.0000043	0.000064
Distilled Water	93.2	
Total	**100**	**100**



30 µl of Nanoparticle Impregnated Edible Film

Figure 24

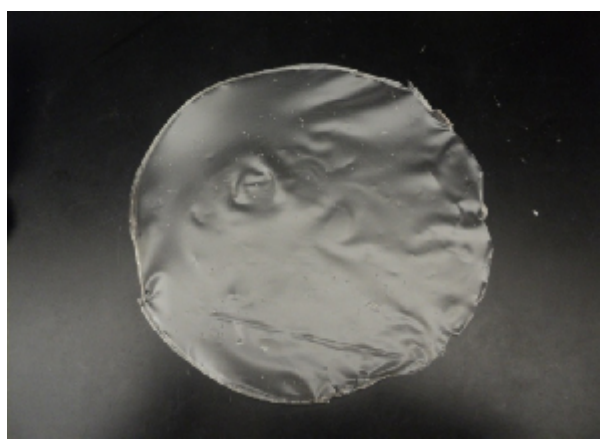
d. Trial 4

Table IV depicts the percentage composition of the edible film impregnated with 60 µl of silver nanoparticles and is depicted in Figure 25.

TABLE IV

PERCENTAGE COMPOSITION OF EDIBLE FILMS (60 µl of Nanoparticle Impregnated Edible film)

Composition Weight (%)		
	Before drying	**After drying**
Tapioca Starch	5	73.52
Glycerol	1.8	26.47
Plant extract	0.0006	0.0088
Silver (60 µl)	0.0000086	0.000128
Distilled Water	93.2	
Total	**100**	**100**



60 µl of Nanoparticles Impregnated Edible Film

Figure 25

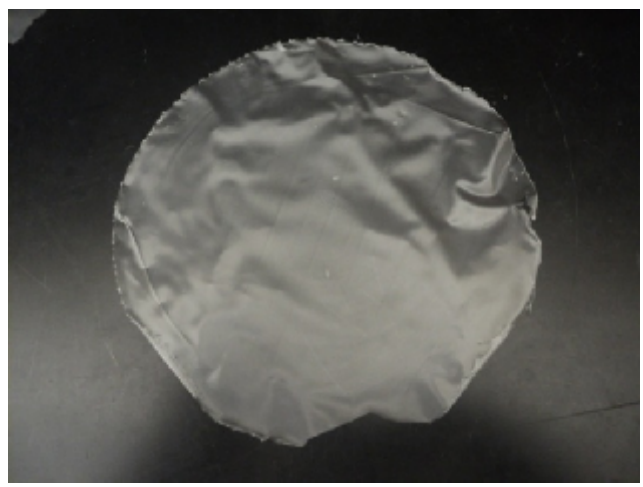
e. Trial 5

Table V and Figure 26 depict the percentage composition of edible film impregnated with 90 µl of silver nanoparticles (Table V).

TABLE V

PERCENTAGE COMPOSITION OF EDIBLE FILMS (90 µl of NanoparticleImpregnated Edible film)

Weight (%)		
Composition	**Before Drying**	**After Drying**
Tapioca Starch	2	73.51
Glycerol	0.72	26.46
Plant extract	0.0009	0.033
Silver (90 µl)	0.000013	0.00048
Distilled Water	97.28	
Total	**100**	**100**



90 µl of Nanoparticle Impregnated Edible Films

Figure 26

f. Trial 6

Table VI and Figure 27 depict the percentage composition of the edible film impregnated with 120 µl of silver nanoparticles.

TABLE VI

PERCENTAGE COMPOSITION OF EDIBLE FILMS

(120 µl of Nanoparticle Impregnated Edible film)

	Weight (%)	
Composition	**Before drying**	**After drying**
Tapioca Starch	2	73.50
Glycerol	0.72	26.46
Plant extract	0.0012	0.044
Silver (120 µl)	0.000017	0.00062
Distilled Water	97.28	
Total	**100**	**100**

120 µl of Nanoparticle Impregnated Edible Films

Figure 27

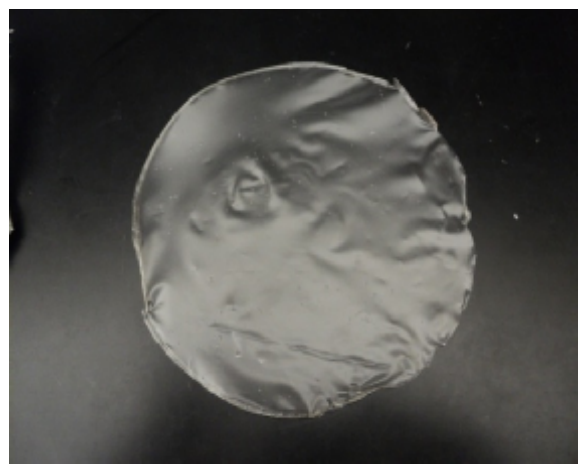
g. Trial 7

Table VII and Figure 28 depict the percentage composition of the edible film incorporated with 150 µl of silver nanoparticles and are given in Table VII.

TABLE VII

PERCENTAGE COMPOSITION OF EDIBLE FILMS (150 µl of Nanoparticle Impregnated Edible film)

	Weight (%)	
Composition	**Before Drying**	**After Drying**
Tapioca Starch	2	73.49
Glycerol	0.72	26.46
Plant extract	0.0015	0.056
Silver (150 µl)	0.000022	0.00081
Distilled Water	97.28	
Total	**100**	**100**



150 µl of Nanoparticle Impregnated Edible Films

Figure 28

The edible film was good with a higher concentration of 150 µl silver nanoparticles. Therefore, the concentration of silver nanoparticles was increased to 0.01%in the next trial.

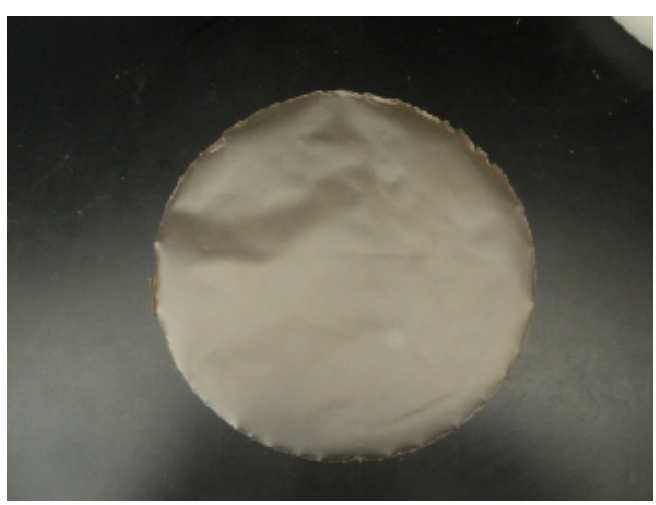
h. Trial 8

Trial 8 consisted of incorporating 3ml Silver nanoparticles, thereby achieving silver concentration of 0.01 per cent as given in Table VIII and Figure 29.

TABLE VIII

PERCENTAGE COMPOSITION OF EDIBLE FILMS
(0.01 % Silver Impregnated Edible film)

Weight (%)		
Composition	**Before Drying**	**After Drying**
Tapioca Starch	2	66.22
Glycerol	0.72	23.84
Plant extract	0.3	9.93
Silver (0.01%)	0.000432	0.01
Distilled Water	96.98	
Total	**100**	**100**



0.01 % Ag Nanoparticle Impregnated Edible Films

Figure 29

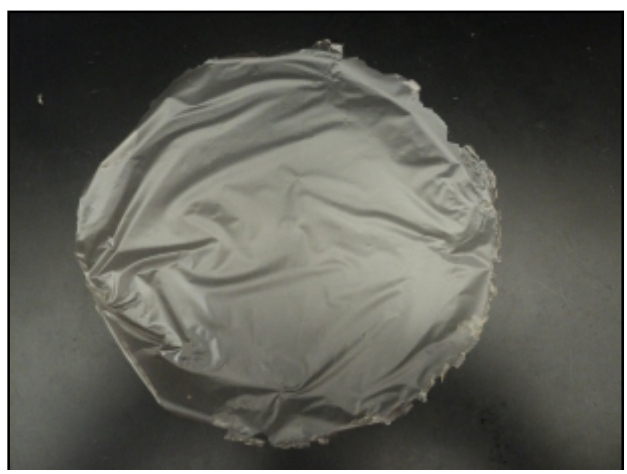
i. Trial 9

Table IX and Figure 30 depict the percentage composition of edible films by incorporating 15 ml silver nanoparticles to achieve a concentration of 0.05 per cent Ag in the edible film.

TABLE IX

PERCENTAGE COMPOSITION OF EDIBLE FILMS
(0.05 % Silver Impregnated Edible film)

Weight (%)		
Composition	**Before drying**	**After drying**
Tapioca Starch	2	47.39
Glycerol	0.72	17.06
Plant extract	1.5	35.55
Silver (0.05 %)	0.00216	0.05
Distilled Water	95.78	
Total	**100**	**100**



0.05 % Ag Nanoparticle Impregnated Edible Film

Figure 30

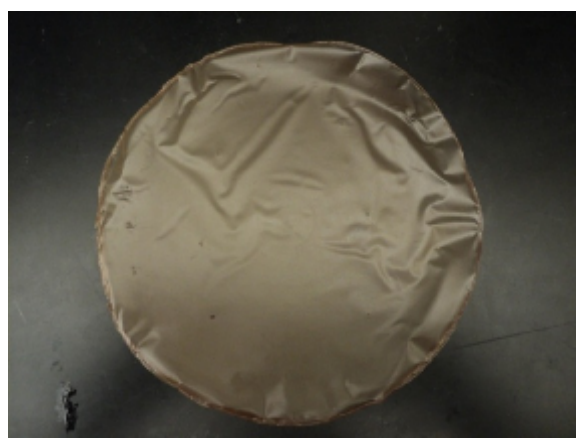
j. Trial 10

The percentage composition carried out by incorporating 60 ml Silver nanoparticles, so as to achieve 0.1 % Ag concentration are given in Figure 31 and Table X.

TABLE X

PERCENTAGE COMPOSITION OF EDIBLE FILMS
(0.1 % Silver Impregnated Edible film

	Weight (%)	
Composition	**Before drying**	**After drying**
Tapioca Starch	2	22.91
Glycerol	0.72	8.24
Plant extract	6	68.74
Silver (0.1%)	0.00864	0.1
Distilled Water	91.27	
Total	**100**	**100**



0.1 % Ag Nanoparticle Impregnated Edible Film

Figure 31

k. Trial 11

The percentage composition of edible films by incorporating 120 ml Silver nanoparticles, so as to achieve a concentration of 0.12 % Ag in the edible filmis depicted in Table XI and Figure 32.

TABLE XI

PERCENTAGE COMPOSITION OF EDIBLE FILMS
(0.12 % of Silver Impregnated Edible film)

Weight (%)		
Composition	**Before drying**	**After dryng**
Tapioca Starch	2	13.57
Glycerol	0.72	4.88
Plant extract	12	81.43
Silver (0.12%)	0.017	0.12
Distilled Water	85.26	
Total	**100**	**100**

0.12 % Ag Nanoparticle Impregnated Edible Films

Figure 32

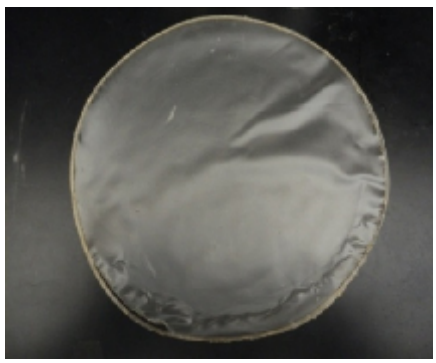
After drying the edible film with 0.12% Ag, it was observed that the film was brittle. Therefore, physical, mechanical and barrier properties of the edible films were tested with the three different concentrations of 0.01, 0.05 and 0.1% Ag respectively.

I. Replicates trials

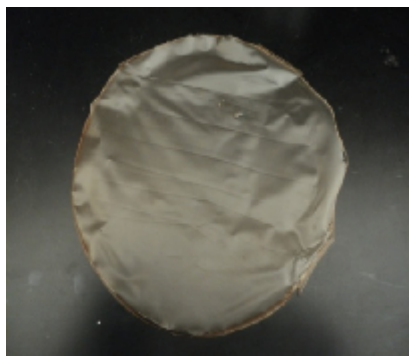
Since trials 8, 9 and 10 involved the highest concentration of Ag they were carried out in replicates with 0.01, 0.05 and 0.1 % Ag impregnated edible films, in order to obtain uniform thickness of the film (Figure 33 and 34).

Before Heating

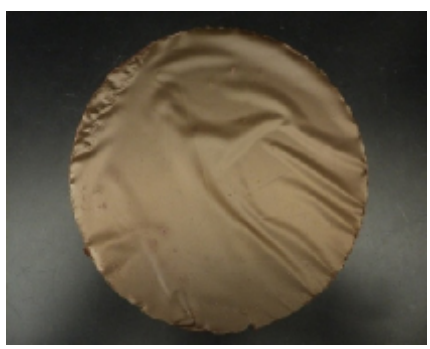
After Heating



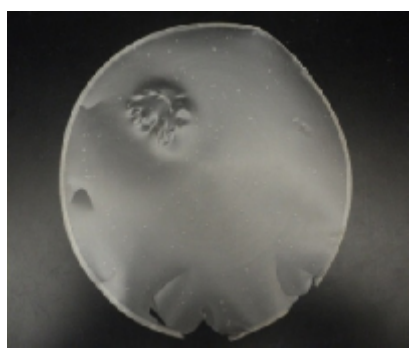
0.01 % Ag



0.05 % Ag



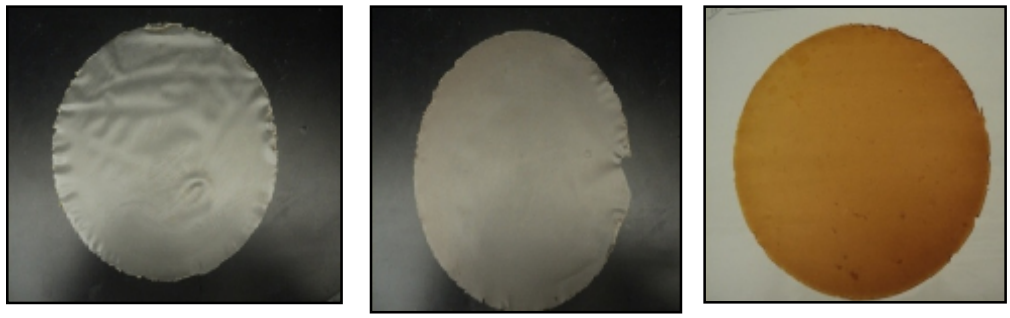
0.1 % Ag



Control

I Set Replicates - After Drying Edible Film

Figure 33



0.01 % Ag **0.05 % Ag** **0.1 % Ag**

II Set of Replicates – After Drying the Edible Film

Figure 34

E. Characterization of Edible Films

1. Thermal Analysis by Differential Scanning

A thermal analytical instrument (Q2000,USA) which is a differential scanning calorimeter equipped with a data collection station was used to scan the thermal transitions of the tested films. The samples were weighed (around 6 mg) using a 5 decimal point XSE Analytical Balance (±0.01 mg) (Mettler Toledo Co. Toledo, OH) in aluminum pans followed by sealing with inverted lids, for optimum thermal conductivity. An empty aluminum pan was also used as a reference, submitted to temperature program, under inert atmosphere (100 ml min^{-1} of Nitrogen). In the first scan, film samples were subjected to cooling from room temperature 0ºC to -20 ºC. In the second scan, heating from - 20ºC to 100ºC. In the third scan, cooling from 100ºC to - 20ºC. In the fourth scan, again subjected to heating from - 20ºC to 250ºC at a rate of 10ºC/min. Nitrogen gas was used to flush the DSC cell at a flow rate of 20 ml/min to maintain an inert environment. Thermograms were recorded and analyzed by the TA Instrument software (Universal Analysis 2000, Version 4.1D). The T_m (melting temperature) was identified as the inflexion point of the baseline.

2. Thermo Gravimetric Analysis

The TGA measurements were made using a thermo gravimetric analyzer (TGA 2920 TA Instruments, New Castle, DE, USA) under a flow of dry Nitrogen (20 ml/min). The films were weighed directly into an aluminum pan (10 mg). The

pans were heated from 30 to 500ºC at a rate of 10ºC/min. The weight loss as function of temperatures was depicted as thermal-gravimetric analysis TGA curve.

3. X-Ray Diffraction

X-ray diffraction measurements (XRD) were performed to evaluate the dispersion of nanoparticles in the starch films. The XRD spectra of the samples were analyzed using X-ray diffractometre (Rigaku Miniflex, Tokyo, Japan) with Cu K α radiation at a voltage of 30 kV and 20 mA. The samples were scanned between 2 ɵ = 3 - 60º with a scanning speed of 2 º/min. Data were collected in a range of 0.08 to 10 º with a step size of 0.01º and a counting of 2s per step. The films were analyzed directly. Bragg's law was used to determine the film interlayer distance $d_{001,}$ where d is the interlayer spacing, λ the wavelength of X-ray and n is a whole number which represents the order of diffraction taken as 1 in our calculations.

4. Water Vapor Permeability (WVP)

The WVP tests were conducted using the ASTM E96 (1996) method with some modifications. Test cups with 50 cm^2 open area were filled with 10g anhydrous calcium sulfate to produce a relative humidity of 0% inside the cup. The film samples were placed on top of the cup and sealed with an O ring. A high vacuum silicone sealant was applied between the O ring and the film samples, between the sealing lip of the cup and the sample, before clamping them with 4 screws. The cups containing the desiccants were weighed to give the initial weight and then placed in a humidity chamber at room temperature (23 ± 2°C) and relative humidity of 55 ± 2%. At half an hour intervals the cups were weighed until a steady state was reached. The water vapor transmission rate (WVTR) through the film was estimated from the linear portion of the plot of weight gained versus time and the slope divided by the film exposure area

$$WVTR = \frac{Q}{t}$$

$$WVTC = \frac{WVTR \times T}{A \times \Delta P}$$

where

A is the surface area (m^2) of the sample exposed to moisture

ΔP is the driving force, describing the humidity difference between two sides of film (Pa).

In this test, Relative Humidity (RH) was determined by measuring dry bulb and wet bulb temperature during the tests and RH value was obtained by using psychometric chart. The driving force ΔP was based on Saturation Pressure of Water Vapor, which corresponded to dry bulb temperature during the test. In this case, water vapor saturation pressure was 7.30×10^3 Pa, according to Saturation Pressure and Temperature Chart.

where

T is the thickness of the film (m)

Q is the weight change of WVT cups (g)

t is the period of time (day)

All tests were conducted in triplicates and the units for WVTC were $gm^{-1}s^{-1}Pa^{-1}$.

5. Antibacterial Testing of Edible Films with Inoculated Bacterial Culture

The Films were cut into small pieces and weighed about 30mg. The films were then exposed to UV light for three hours to eliminate the possible contamination that may have occurred during cutting and transferring process. The tryptic soy broth of about 20ml was transferred to each test tube and inoculated with 10^2cfu/ml. The amount of bacterial culture in each tube was tested on edible films with silver nanoparticles at three different concentrations of 0.01, 0.05 and 0.1% silver along with the control. After adding proper amount of bacterial culture in each test tube, all tubes were placed in an incubator aerobically at 37°C for 24 hours (for *E.coli, S.enterica and S.dysenteriae)* respectively. The O.D. readings were periodically tested for every three hour.