V. SUMMARY AND CONCLUSION

Food borne diarrhea is becoming a major public health problem with higher morbidity and mortality rate among the people and food safety is becoming a world wide issue due to the presence of pathogenic bacteria in foods. Highly perishable foods are posed for food spoilage due to enzymes, microbes and intrinsic conditions, which are characterized by reduced shelf life, increased microbial counts and loss of sensory attributes which make the food unfit for human consumption. Apart, from microbial contamination, oxidation is another crisis in spoilage of packaged foods. As a result, diarrheal incidence among children below five years of age are more rampant in India, than other countries in the world.

Silver nanoparticles are popular as nanocoating in food packages. They are impregnated as an edible film to ensure food preservation. The European Food Safety Authority (2011) have studied the toxicological effects of nanosilver with laboratory animals for 30 – 90 days, which proved to have similar results observed in dietary studies with silver salts. No toxic effect have been observed with nanosilver. Further, the migration levels of silver into food was 0.05 mg/Kg of Ag into the food i.e. below the European Specific Migration Limit (SML) that have proved to lower the risk of adverse effect of silver with low consumer exposure (Toxconsult, 2016).

According to Indian Herbal Pharmacopoeia (2010), medicinal plants are widely used as anti inflammatory, antistress, antiulcer, anti diabetic and antidepressive agents. Incorporating medicinal plants onto nanocoated food packages could provide greater food safety. Four medicinal plants were analyzed for various potentials for incorporation as edible coats. Of these *Glycyrrhiza glabra*was chosen for the study since there were ample evidences from literature that can act as major sources of antioxidants. They possess antimicrobial activity against the enteropathogenic species. The AgNP synthesized from medicinal plants are rich in phytoconstituents posing no toxic effects and are ecofriendly in nature. These nanoparticles are embedded as a nanocoating onto the polymer such as PET, PP and PE (Simon *et al.,* 2008).

There is a need for synthesizing silver nanoparticles (AgNP) from medicinal plants to promote safety in food processing. The present study is a step in this direction, wherein AgNP coats from four medicinal plants were undertaken. The best among them (coats and films) from *Glycyrrhiza glabra* were employed in this study.

The present study was undertaken with the following objectives to

1. Optimize Silver nanoparticles from four medicinal plants
2. Identify the Silver nanocoat from medicinal plants with highest antioxidant, antimicrobial and anticancer potentials
3. Coating of AgNP (*Glycyrrhiza glabra*) onto three food packages namely PET, Infant feeding bottles (PP) and Zip Loc covers (PE)
4. Develop food containers with longer inhibition for diarrheal causing bacteria by employing plant extracted with silver nanoparticles
5. Develop food packaging containers for sustained sterility over longer duration using silver nanoparticles
6. Investigate the thermal, barrier and antimicrobial properties of Edible Films.
7. Develop the edible films of *Glycyrrhiza glabra* AgNP, which could serve as a value added product

The findings of the study are summarized below :

Phase I

Synthesis and Optimization of Silver Nanoparticles

- Four medicinal plants were utilized to synthesize silver nanoparticles by optimization methods through ultracentrifugation (1 hr 10 minutes). Of these, *Leucas aspera* produced silver nanoparticles with a particle size of 23.44 nm and a suspension stability of -24.7 mV. Hence, the other ultra centrifugation (1 hr 10 minutes) with a g value (12,000) was adopted as the standardized method for synthesizing the AgNP from other medicinal plants.

- Among the four medicinal plans, it was observed that *Glycyrrhiza glabra* AgNP showed a smaller particle size of 34.7 nm with highest suspension stability of -28.9 mV, compared to other AgNP of plant extracts.

Antioxidant and Antimicrobial Quality of Phytochemical Constituents

- Of the four medicinal plants, *Glycyrrhiza glabra* had the maximum antioxidant potential of 520 µg and *Leucas aspera* had the minimum antioxidant potential of 132 µg respectively. The highest antioxidant content of *Glycyrrhiza glabra*could be due to presence of phytoconstituents in the plant extract.
- At all concentrations of AgNP synthesized from the four medicinal plants, *Glycyrrhiza glabra* AgNP had the highest antibacterial activity. By agar well disc diffusion technique, it was found that of the varied concentrations of *Glycyrrhiza glabra* AgNPshowed that 30 µl had the maximum zone of inhibition (11mm), compared to the control (3mm) against *E.coli (MTCC 40).* Larger inhibitory zones of 15mm than control (3 mm) against *S. enterica (MTCC 3219)* and 14 mm than control (4 mm) against *Sh.dysenteriae (PSGIMS & R)* were observed.
- Mean ±SD values of the control (*Glycyrrhiza glabra*extracts) showed 3 ± 0.35, 2 ± 0.21 and 4 ± 0.26 against *E.coli (MTCC40), S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R)*the visible bacterial growth within 24 hours of the study was observed. The differences in the control samples for all the microbes were statistically not significant.
- Out of silver nanoparticles synthesized from the four medicinal plants, *Glycyrrhiza glabra* had the maximum antibacterial activity by total viable counts. It was observed that among different concentration of nanoparticle, 30 µl of *Glycyrrhiza glabra* AgNP had the maximum antimicrobial activity of 64.18 per cent against *E.coli (MTCC 40),* 62.92 per cent *against S.enterica (MTCC 3219)* and 62.16 per cent *against Sh.dysenteriae (PSGIMS & R)* respectively. The control (*Glycyrrhiza glabra*) showed 67 ± 3.67, 89 ± 5.84 and 74 ± 3.25 against *E.coli (MTCC40), S. enterica (MTCC 3219)*

and*Sh.dysenteriae (PSGIMS & R)*. It was observed that these values were statistically not significant.

Characterization of *Glycyrrhiza glabra*Silver Nanoparticles

- By GC/MS, the major compounds found in *Glycyrrhiza glabra* were 9Octadecenal (RT=14.75), cis 9,10 Epoxyoctadecan-1-ol(RT =17.34) and Z,Z,2,5-Pentadecadien-1-ol (RT=20.71) are the major aldehyde and alcoholic compounds which possess the antimicrobial activity. The most dominant fatty acids were tetradecanoic acids (RT=11.58), n hexadecanoic acid (RT=12.84) which are capable of scavenging free radicals, due to their antioxidant property. In addition, these phytocompounds of *Glycyrrhiza glabra*may act as a capping and reducing agent in synthesizing silver nanoparticles.
- The phytochemical screening of *Glycyrrhiza glabra* AgNP by Thin Layer Chromatography (TLC) showed the presence of phenolic compounds with a purple colour. The FTIR confirmed the presence of phenols, which showed a narrow and sharper peak at 578.06 cm^{-1}, which could be one of the reason for its maximum antioxidant potential.
- Among the FTIR spectra of the four medicinal plants, *Glycyrrhiza glabra* raw extracts (Before Reaction with $AgNO_3$) and AgNP (After Reaction with $AgNO_3$) showed the characteristic band at 3396.90 cm^{-1} and 3410.10 cm^{-1} due to the coupled alcohol OH stretch and NH amine stretching groups.
- FTIR of *Glycyrrhiza glabra* root and colloids showed the functional peak such as C=C, C-O-C, C=O, C-O and C-N corresponding to biomolecules like phenols, flavonoids and terpenoids, which are involved in reduction and capping of silver ions leading to effective chelation of silver nanoparticles.
- Greater interaction of water with silver could be the reason for OH stretching at 3410.10 cm^{-1} involved in capping of AgNP. In both the raw and AgNP *(Glycyrrhiza glabra),* the vibration bands at 1644.90 cm^{-1} with a shift to 1627.87 cm^{-1} indicate the presence of carbonyl groups in the NH amide linkages of protein.

- By EDX, the elemental analysis of *Glycyrrhiza glabra* AgNP showed that the silver content is about 0.64 per cent, which act as a potent chelating agent with the phytochemicals of the plant extract. The highest level of cationic impurity namely sodiumof 41.75 per cent and anionic impurity such as chlorine and sulphur were 35.78 per cent and 1.86 per cent respectively. The higher percentage of impurities of Na and Cl of *Glycyrrhiza glabra* AgNPcould be due to the soil rich in minerals, which may vary based on the geographical location.
- The SEM of *Glycyrrhiza glabra* AgNP showed spherical shaped homogenously distributed with an average grain size of 617.57 nm, as observed from the magnification of (X 20,000). The TEM of *Glycyrrhiza glabra*AgNP showed polydispersed colloidal particles with a grain size of 304.68 nm. The high resolution TEM images indicatesgood crystallinity of the nanoparticles.
- From MTT assay, it was found that 200 µl of *Glycyrrhiza glabra* AgNP showed 50 percent and 48 percent apoptosis in heLa and liver carcinoma cell lines than the control. The apoptosis of cancer cells which could be to the cytotoxic effect of *Glycyrrhiza glabra*AgNP. In addition to antimicrobial property*, Glycyrrhiza glabra* possess the anticancer activity.
- By Gel electrophoresis, *Glycyrrhiza glabra*AgNP showed a longer DNA damage with heLa and Liver carcinoma cell lines compared with to the control and marker. It was observed that the DNA fragmentation increased on exposure to*Glycyrrhiza glabra*AgNP.

Phase II

Coating of Silver Nanoparticles *(Glycyrrhiza glabra*) onto Commercially Available Food Packages

- Module 1 revealed that of the different concentrations, 30 µl of *Glycyrrhiza glabra* AgNP had the smallest particle size, stable zeta potential with highest antioxidant and antimicrobial potential. Hence, 30 µl of *Glycyrrhiza glabra* nanoparticles was selected as the best colloidal solution for coating onto the food packaging material such as PET, infant feeding bottles (Poly Propylene) and Zip lock covers (Poly Ethylene).

- Determination of antimicrobial activity of *Glycyrrhiza glabra* AgNP at varied concentrations from 10 to 40 µl and 50 to 125 µl of nanocoated PET materials (experimental samples) and uncoated (control) against the enteropathogenic species showed 58.93 per cent of inhibition against E*.coli (MTCC 40)*, 46.67 per cent of inhibition against *S.enterica (MTCC 3219)* and 41.67 per cent of inhibition against *S.dysenteriae (PSGIMS&R*). Thecontrol samples showed the highest microbial growth. Statistical analysis between the experimental (nanocoated) and control(uncoated) PET bottles showed a statistically significant ($P<0.01$) decrease in microbial growth in the former compared to the latter.
- Shelf life study of nanocoated PET bottles stored with foods such as tomato puree and lemon juice were studied against the three enteropathogenic diarrheal species.

Shelf Life Study of Nanocoated PET bottles with tomato puree

- It was observed that the nanocoated PET bottles from 0 to 6th day showed no microbial growth and on 8th day had 57.52 per cent of microbial inhibition. On 10th day, the inhibition reduced to 56.83 per cent against the *E.coli (MTCC 40)* than the control (uncoated) which exhibited no microbial inhibition.
- Similarly, from 0day to 6th day, no microbial growth was observed against *S.enterica (MTCC 3219).* However on the 8th day, nanocoated PET bottles showed 57.52 per cent inhibition which got reduced to 56.83 per cent on 10th day against the *S.enterica (MTCC 3219*) while the control samples showed no inhibition whatsoever
- No microbial growth was observed from 0day to 6th day in nanocoated PET bottles against *Sh.dysenteriae (PSGIMS & R).* On 8th day and 10th day of storage, there was an increase in inhibition of 87.5 per cent to 87.66 per cent against the *S.dysenteriae (PSGIMS & R)* respectively. The difference

between the efficacy of nanocoated and uncoated PET bottles interms of microbial growth inhibition was statistically significant (P< 0.01).

Shelf Life Study of Nanocoated PET bottles with Lemon Juice

- The shelf life study of nanocoated PET bottles stored with lemon juice against enteropathogenic species.
- It was found that nanocoated PET bottles with lemon juice showed no bacterial growth till 8th day, compared to the control (uncoated) PET bottles against *E.coli (MTCC 40)* and *S.dysenteriae (PSIMS & R)* respectively. On 10th day, nanocoated PET bottles with lemon juice showed 77.67 per cent of microbial inhibition against the *E.coli (MTCC 40).*
- From 0 to 10th day, the nanocoated PET bottles with lemon juice showed no microbial growth against *S.enterica (MTCC 3219),*while the control (uncoated) PET bottles showed TNTC (Too Numerous To Count). The difference between nanocoated and uncoated PET bottles was statistically significant (P< 0.01).
- Similarly, Nanocoated PET bottles with lemon juice revealed that from 0day to 6th day, showed no microbial growth while control (uncoated) PET bottles had ahigher microbial growth *S.dysenteriae (PSGIMS &R)*. On 8th day, it was found that 91.5 per cent of inhibition decreased to 87.67 per cent, when compared to the control (uncoated) against *S.dysenteriae (PSGIMS &R)*.
- The difference between the experiment (nanocoated) and control (uncoated) PET bottles stored with lemon juice was found to be significant at P< 0.01

Shelf Life Study of Nanocoated Infant Feeding Bottles (PP) with Milk

- Varied concentrations of *Glycyrrhiza glabra* AgNPs from 10 to 40 µl, 50 to 125 µl with PP material were tested against control (uncoated) for its shelf life stability of milk and lemon juice were tested against *Sh.dysenteriae (PSGIMS & R)*for every ½ an hour for 3 hours against *S. dysenteriae (PSGIMS &R)*.
- From 0 – 1 ½ hour, nanocoated PP feeding bottles with *Glycyrrhiza glabra* AgNP showed no microbial growth, when compared to the control (uncoated)feeding bottles against*S.dysenteriae (PSGIMS & R)*.

- At 2hrs of storage of milk, nanocoated feeding (PP) bottles showed 92.33 per cent of microbial inhibition against *S.dysenteriae (PSGIMS& R).* At 2½ and 3 hour, the inhibitory activity decreased from 86.33 per cent and 84 per cent, compared to the control (uncoated) PP feeding bottles which showed no inhibiton against *S.dysenteriae (PSGIMS &R).*

Shelf Life Study of Nanocoated Infant Feeding Bottles (PP) with Lemon Juice

- In nanocoated PP feeding bottles stored with lemon juice, there was no microbial growth from 0 to 3 hours, while the control (uncoated) had TNTC microbial counts in feeding bottles.
- Therefore, the experimental (nanocoated) infant feeding bottles (PP) stored with lemon juice showed maximum inhibitory activity against *Sh. dysenteriae* (PSGIMS & R).

Shelf Life Study of Nanocoated Zip Loc covers (PE) with Tomato Puree

- By spread plate method, the varied concentration of *Glycyrrhiza glabra* AgNPs from 10 to 40 µl and 50 to 125 µl along with Zip Loc (PE) material and control (only PE material) were tested against the enteropathogenic species.
- Nanocoated zip loc covers with varied concentrations of AgNP showed that 30µl had maximum inhibiton of 54.55 per cent against *E.coli (MTCC 40)* and 73.42 per cent inhibition against S.enterica (MTCC 3219) when compared to the control (uncoated). The difference between the experiment (nanocoated) and control (uncoated) zip loc covers (PE) was found to be statistically significant ($P<0.01$).
- The shelf life study of nanocoated zip loc covers (PE) stored with tomato puree were tested against the enteropathogenic species for a period of ten days.
- From 0th day to 6th day, the nanocoated zip loc covers (PE) stored with tomato puree showed no microbial growth against the *E.coli (MTCC 40),* when compared to the control (uncoated) zip loc covers. On 8th day, the

nanocoated zip loc covers showed 84 per cent inhibition and on 10th day the inhibition declined to 56.83 percent, which could be due to higher moisture content of tomato puree that increased the microbial load.

- From 0thto 6th day, there was no microbial growth in nanocoated zip loc covers, compared to the control (uncoated) which showed a higher microbial count against the *S.enterica (MTCC 3219)*. From 8th to 10th day, nanocoated zip loc covers had 73.53 per cent of microbial inhibition, which decreased to 57.57 per cent against *S.enterica (MTCC 3219)*compared to the control (uncoated) zip loc covers until ten days of storage time.
- No microbial growth was observed from 0day to 6th day in nanocoated covers, when compared to the control (uncoated) against the *S.dysenteriae (PSIMS & R).* On 8th day, nanocoatedzip loc covers showed 91.5 per cent inhibition and on 10th day, its inhibitory activity declined to 87.67 per cent against *S.dysenteriae (PSGIMS & R)* respectively. The difference between the experimental (nanocoated) and control (uncoated) was statistically analyzed and are found to be significant at ($P<0.01$) .
- It was observed that the tomato puree stored in nanocoated zip loc covers showed the maximum inhibition of 87.67 per cent than the control (uncoated) against the *S.dysenteriae (PSGIMS & R)* until astorage period of ten days. These silver nanoparticles were found to be highly effective against the diarrheal species. Therefore, these nanoparticles coated onto surface of the food packages could inhibit the growth of microbial pathogens.

Phase III

Synthesis, Standardization and Characterization of Nanogranular Edible Film

- As an alternative to synthetic polymers, in the present study, synthesis and optimization of eco friendly edible *Glycyrrhiza glabra* AgNP impregnated starch films at three different concentrations of 0.01, 0.05 and 0.1 % silver with *Glycyrrhiza glabra* nanoparticles were tested for its thermal, barrier and antibacterial activity
- The thickness of the Ag impregnated starch films were calculated from five readings taken from the films. The thickness of the filmranged 0.3 mm for

0.01 per cent Ag films, 0.4 mm for 0.05 and 0.1% Ag impregnated films. These values were statistically analysed and the differences were statistically not significant.

- The moisture content of edible film decreased from 9.65 to 5.14 %, with the increase in impregnation of silver from *Glycyrrhiza glabra* Ag NP of 0.01 % Ag to 0.1 % Ag of the edible film. This may be due to specific interactions that are linked to hydroxyl groups in starch and nanoparticle polymer water interactions by hydrogen bonding, which caused a decrease in moisture content of these edible films.
- The WVP of the tapioca film without nanoparticles (control) showed the highest water vapour transmission of 6.60 X 10^{-11}g $\times m^{-1}s^{-1}Pa^{-1}$. The edible film incorporated with increased concentration of nanosilver at 0.01%, 0.05 and 0.1% Ag showed a decreased WVP values such as 5.65 X 10^{-12}, 4.24 X 10^{-12} and 3.63 X 10^{-12} $g\times m^{-1}\times s^{-1}\times Pa^{-1}$ respectively, when compared to thecontrol (tapioca starch) was 6.60 x 10^{-11} $gxm^{-1}s^{-1}Pa^{-1}$. A significant decrease ($p<0.05$) was observed with the addition of Ag nanoparticles, when compared to the control (tapioca starch).
- Thermally induced endothermic transitions were observed for edible films incorporated with silver nanoparticles at three different concentrations of 0.01, 0.05 and 0.1% of *Glycyrrhiza glabra* AgNP. Increase in silver concentration in the edible film showed a melting enthalpy of the edible film of 0.01, 0.05 and 0.1 % Ag indicating a crystalline nature namely 59.06, 27.50, 71.88 (ΔH) J/g respectively, when compared to the control (tapioca starch) enthalpy of 113.70 (ΔH) J/g. Lower enthalphy was observed, compared to the control. However, the silver concentration in the films do not have straight correspondence. There was no change in glass transition temperature (T_g) corresponding to the concentration of Ag nanoparticles impregnated starch film.
- Of the different concentration of silver nanoparticles impregnated edible film, the TGA results of 0.01% edible film was found to be the best film, which exhibited degradation at 370ºC. The values were value of the control (380ºC). The TGA of silver nanoparticles impregnated edible film of 0.05 and

0.1 per cent was 390ºC due to the incorporation of increased nanoparticles, which increased the degradation temperature between the nanoparticles and biopolymer matrix (starch) of the edible nanogranular film. The pyrolysis of these Ag impregnated edible film took place between 300 ºC to 400 ºC respectively.

- These degradation events may be due to loss of functional groups, back bone fragments and are finally charred. Thus, the initial thermal decomposition temperature shifted to a higher temperature by increasing the silver nanoparticle concentration in the Ag impregnated tapioca starch film. Thus, in the present study, the incorporation of silver impregnated edible films improved the thermal stability of the films, which may be due to increased dissociation energy related to the intentsified interactions between the nanoparticles and starch, when compared to the tapioca starch (control).
- From XRD of *Glycyrrhiza glabra* AgNP presence of amylose in all the film of 0.01, 0.05 and 0.1 per cent Ag impregnated tapioca starch film with characteristic peaks at $2\theta = 18^\circ$ and 20° corresponds to single helical conformation. In addition, diffractogram also confirms the presence of amylopectin in all the film of 0.01, 0.05 and 0.1 per cent Ag impregnated tapioca starch film. Characteristic peaks at $2\theta = 16^\circ$ and 19° confirm the amorphous nature and pseudo crystalline structure. In the present study, an increase in crystallinity of these Ag nanoparticle impregnated film of 0.01 and 0.05 per cent may be due to the OH groups of amylose in tapioca starch, which improved the interaction with nanoparticles and glycerol.
- Among the three different concentrations of 0.01, 0.05 and 0.1 per cent Ag impregnated nanofilms, it was observed that 0.01 per cent Ag film had the highest antimicrobial activity against *E.coli.*

The findings of the study reveal that the Silver nanoparticles (*Glycyrrhiza glabra)* are highly effective against the diarrheal pathogens, when coated onto food packages namely PET, Infant feeding bottles (PP) and Zip Loc covers(PE), stored with acidic and alkali foods. In addition, these nanoparticles were impregnated onto tapioca starch which produced edible film with improved thermal, barrier and antimicrobial property against the diarrheal species.

Therefore, both the nanocoating and edible film are more potent against the diarrheal species which could be due to synergisitc action of both the antioxidant and antimicrobial *property of Glycyrrhiza glabra* silver nanoparticles. It also increases the shelf life of foods against diarrheal pathogens ensure total food safety and are ecofriendly in nature.

Based on the findings of the study, the following recommendations are made :

1. Identify any other active components in *Glycyrrhiza glabra root* extract using various other solvents
2. Study the effect of migration of silver from nanocoated packages into foods
3. Shelf life study on foods in nanocoated packages beyond six months
4. The changes during recycling of nanocoated food packages after use
5. Study the antimicrobial effect of *Glycyrrhiza glabra* silver nanoparticles against diarrheal pathogens in foods stored at refrigeration temperature.
6. Perform similar studies on foods packed in other food packages such as PVC, Aluminium foils, etc.