IV - RESULTS AND DISCUSSION

The results of the present investigation titled **“Synthesis of Eco-friendly Nanogranular Films in Food Packaging using Medicinal plants and Nanoparticles”** are presented and discussed under the following headings:

Phase I

A. Synthesis, Characterization and Antimicrobial Property of Silver Nanoparticles

1. Authentication of Medicinal Plants from Botanical Survey of India (BSI)
2. Comparison of Conductivity of Selected Medicinal Plants
3. Characterization of Plant Extracts with Silver Nanoparticles
4. Antioxidant and Antimicrobial Quality of Phytochemical Constituents

Phase II

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

1. To assess the antimicrobial property and shelf-life of nanocoated food packages
   a. PET (Poly Ethylene Terephthalate)
   b. Infant feeding bottles (PP)
   c. Zip lock covers (PE)

Phase III

C. Synthesis, Standardization and Characterization of Nanogranular Edible Films

1. Synthesis and Standardization of Silver nanoparticles (*Glycyrrhiza glabra)* Edible films
2. Characterization of Edible Films

Phase I

A. Synthesis, Characterization and Antimicrobial Property of Silver Nanoparticles

1. Authentication of Medicinal Plants from Botanical Survey of India (BSI)

The Authentication of Medicinal Plants was obtained from Botanical survey of India, Tamil Nadu Agricultural University, Coimbatore and the certificate needs as follows :

1. The plant specimen was identified as *Andrographis paniculata* (Burm.F.) Wall.ex. Nees – ACANTHACEAE with BSI No: BSI/SRC/5/23/2012-13/Tech- 739

2. The plant specimen was identified as *Leucas aspera*– LAMIACEAE with BSI No: BSI/SRC/5/23/2012-13/Tech- 1167

3. The plant specimen was identified as *Curcuma longa* – ZINGIBERACEAE with BSI No :BSI/SRC/5/23/2012-13/Tech- 823

4. The plant specimen was identified as *Glycyrrhiza glabra*– FABACEAE with BSI No :BSI/SRC/5/23/2012-13/Tech - 947

2. Comparison of Conductivity of Selected Medicinal Plants

Table XII and XIII indicate that the prepared suspension of *Leucas aspera*AgNP preserves the conductivity over a period of time reflecting that the Ag nanoparticles are likely to be encapsulated by the plant extract and that protects the activity of the particles over 48 hrs.

TABLE XII

CHANGES IN CONDUCTIVITY OF LEUCAS ASPERA EXTRACT

CentrifugeTime (minutes)	Viscosity of the dispersion medium (mPa.s)	Conductivity (ms/cm)
GLC -10 min	0.8872	0.434
GLC -20 min	0.8861	0.480
GLC - 30 min	0.8872	0.508
GLC - 60 min	0.8896	0.511
Ultra centrifuge- 15 min	0.8930	0.340
Ultra centrifuge- 45 min	0.8926	0.374
Ultra centrifuge- 1 hr 10 min	0.8948	0.470

TABLE XIII

COMPARISON OF CONDUCTIVITY OF SELECTED MEDICINAL PLANTS

Name of the Medicinal Plants	Viscosity of the dispersion medium (mPa.s)	Conductivity (ms/cm)
Andrographis paniculata	0.895	0.493
Curucuma longa	0.880	0.323
Glycyrrhiza glabra	0.896	0.528
Leucas aspera	0.895	0.470

Table XII and XIII compares the conductivity of selected medicinal plants. It was observed that the conductivity of AgNPs from four medicinal plants are of considerable importance while synthesizing these nanoparticles. The Ag nanoparticles synthesized from four medicinal plants, showed that there a slight change in conductivity, which may be due to ion dissociation from the nanoparticle surface.

Rao *et al.,* (2013) state that higher value of the zeta potential confirms repulsion among the nanoparticles, thereby increasing stability of the suspension.

The zeta potential value of the AgNP, which could be positive or negative.(Edison *et al*., 2013) described that the negative zeta potential of AgNPs, which could be due to the possible capping of the bio-organic components present in the extract. The higher values of z potential showed greater electrostatic repulsion between the nanoparticles, thereby lowering the agglomeration in the suspension.

3. Antioxidant and Antimicrobial Quality of Phytochemical Constituents

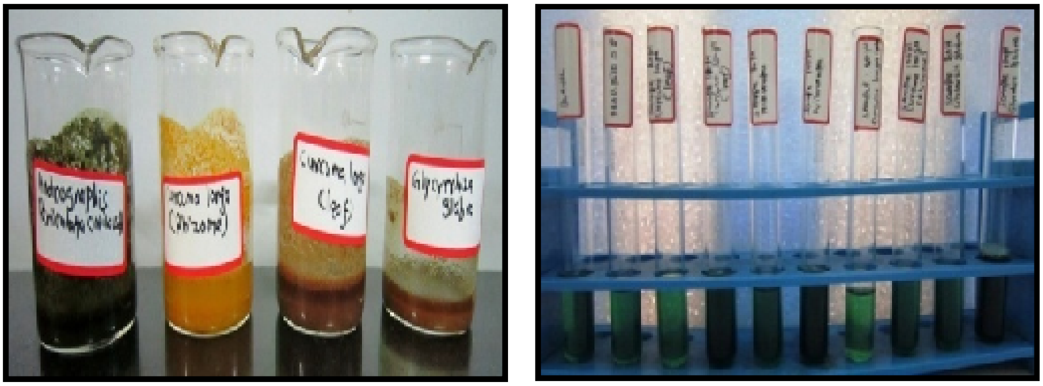
a. Antioxidant Activity

Table XIV depicts the FRAP Antioxidant activity of Silver Nanoparticles from the four medicinal plants.

TABLE XIV

ANTIOXIDANT ACTIVITY OF MEDICINAL PLANTS

Name of the Medicinal Plants	Concentration (µg)
Leucas aspera	132
Curcuma longa	395
Glycyrrhiza glabra	520
Andrographis paniculata	446



Antioxidant Activity of Medicinal Plants

Figure 35

Glycyrrhiza glabra had the maximum antioxidant potential of 520 µg, when compared to *Andrographis paniculata and Curcuma longa.* It has been observed that *Leucas aspera* had the minimum antioxidant potential of 132 µg respectively (Figure 35). Of the four medicinal plants, *Glycyrrhiza glabra* showed the highest antioxidant activity and was selected for the study. The highest antioxidant content could be due to presence of phytoconstituents in the plant.

Cheel *et al.,* (2010) report that the aqeous extracts of *Glycyrrhiza glabra* are more effective than the isolated compounds of *Glycyrrhiza glabra.* Kaur *et al.,* (2013) describe that *Glycyrrhiza glabra* shows a higher antioxidant potential, which is contributed by the presence of abundant phenolic compounds such as liquiritigenin apiosylglucosides. In addition, other bioactive antioxidants of *Glycyrrhiza glabra* are coumarins, stilbenoids, saponins, flavanoids, isoflavones and its derivatives such as glabridin, hispaglabridin A, B, 4'-O-methyl glabridin, echinatin, chalcones and licochalcones A,B,C and D, which would act as a free radical scavengers, thereby preventing the lipid oxidation and peroxidation process. Sharma *et al.,* (2013) state that the *Glycyrrhiza glabra*contains major phytoconstituents like alkaloids, phenolics, saponins and flavonoids exhibiting highly effective antioxidant activity, which would fight against the scavenging free hydroxyl radical and microbes.

Ali *et al.,* (2013)suggest that the phytoconstituents of *Glycyrrhiza glabra* are rich in antioxidants, which could be used as food additives to increase the shelf life of foods. Jiang *et al.,* (2013) report that the efficiency of water extracts (*Glycyrrhiza glabra)* at a low concentration showed a maximum antioxidant property, thereby inhibiting lipid oxidation and enhancing the sensory qualities of food. Rubio *et al.,* (2013) report that phenolic compounds of *Glycyrrhiza glabra* are the major antioxidants, because of their singlet oxygen quenching effect, metal chelation and free radical scavenging activity, thereby preventing lipid peroxidation by forming the pro-oxidant molecules (cytokines, 5 LOX and leukotrienes). Several research studies have evidenced that medicinal plants are the natural sources of active phytochemicals with antioxidants, thereby neutralizing the free radicals

Several research studies show that antioxidants from medicinal plants are highly effective in preventing the oxidation of foods, thereby reducing food borne diseases and enhancing the shelf-life of foods (Santhi *et al.,* 2011). Roots of *Glycyrrhiza glabra*(roots)possess a broad variety of polyphenolic compounds (amphipathic) in nature, thereby preventing the food borne diseases (Ivanova *et al*., 2005). The major antioxidants of *Glycyrrhiza glabra* are phenols, flavonoids, tannin, alkaloid, glycosides and saponins, which are found to be active against bacteria (Scalbert, 1991).

Gaitry*et al.,* (2013) describe that free radical scavenging activity of *Glycyrrhiza glabra* extracts show a maximum antioxidant activity of 67.22 percent at 500µg/ml, due to the presence of sugars, flavonoids and saponins. Singh *et al*., (2010) elucidate that the *Glycyrrhiza glabra* possesses a considerable amount of antioxidative enzymes and phytochemicals, which are attributed to its antimicrobial property. Hence, these plant extracts are used as food preservative to increase the shelf life of stored foods. Jadhav *et al.,*(1996) opines that the natural antioxidants of *Glycyrrhiza glabra* (roots) are phenols, carotenoids, flavonoids, vitamins and dietary glutathione. It is also reported that the antioxidants of these plant extracts, when added to foods, prevent the oxidative rancidity, thereby increasing shelf life and quality of foods.

b. Antimicrobial Activity of Silver Nanoparticles in the Presence of Plant Extracts

Based on p78article size, zeta potential and antioxidant activity, among four medicinal plants, *Glycyrrhiza glabra* showed the maximum antioxidant potential with smaller particle size and a highly stable zeta potential. Hence, the suspension of G*lycyrrhiza glabra* AgNPs was selected as the excellent nanoparticle suspension, when compared to nanoparticles suspension from the other three plant extracts.

The synergistic effect of medicinal plant extracts from the four medicinal plants encapsulated with silver were analysed to compare the effect of concentration of AgNP at varied concentration from 10, 20, 30 µl (chelated with plant extracts) and control (only plant extracts) were tested against enteropathogenic species namely *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R),* which were determined using the standard agar well disc diffusion to determine the antimicrobial activity of the silver nanoparticles from plant extracts.

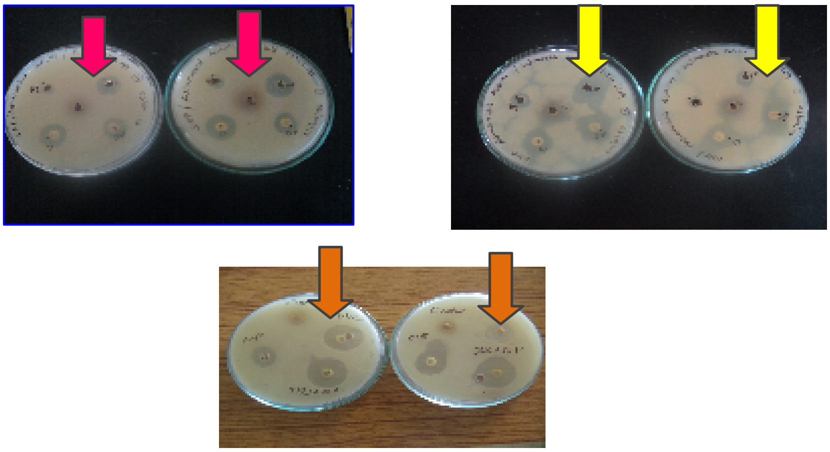
Table XV shows the antimicrobial activity of Silver nanoparticles from the four medicinal plant extracts

TABLE XV

ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES FROM FOUR MEDICINAL PLANTS

Silver Nanoparticles (AgNP) of Medicinal Plants	**Zone of Inhibition (mm)**			
	E.coli (MTCC 40)	***S.enterica (MTCC 3219)***	***Sh.dysenteriae (PSGIMS &R)***	**Plant extracts (Control)**
Leucas aspera - 10 µl	2	4	3	1
Leucas aspera - 20 µl	3	6	5	1
Leucas aspera - 30 µl	6	8	7	2
Andrographis paniculata - 10 µl	2	2	3	1
Andrographis paniculata - 20 µl	4	3	5	1
Andrographis paniculata - 30 µl	7	6	10	3
Curcuma longa - 10 µl	4	5	4	1
Curcuma longa - 20 µl	5	7	7	1
Curcuma longa - 30 µl	8	10	12	2
Glycyrrhiza glabra - 10 µl	9	8	5	1
Glycyrrhiza glabra - 20 µl	13	12	9	1
Glycyrrhiza glabra - 30 µl	11	15	14	4

Figure 36 to 39 depicts the antibacterial activity of AgNP of four medicinal plants against *the E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS &R).*



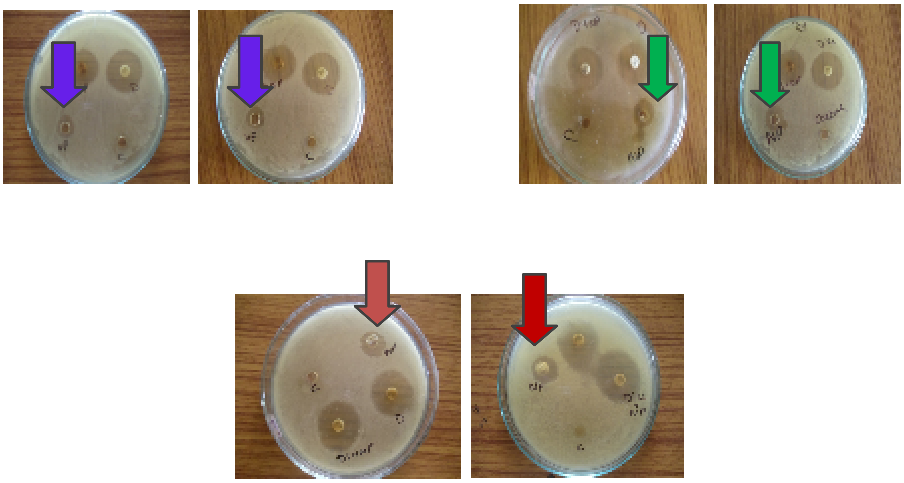
A – *E.coli*

B – *S.enterica*

C – *Sh.dysenteriae*

Antibacterial Activity of *Leucas Aspera* AgNP

Figure 36



A - *E.coli*

B – *S.enterica*

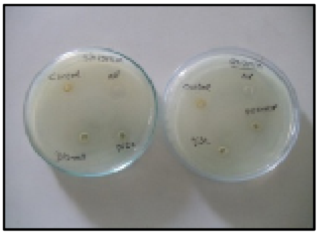
C– *Sh.dysenteriae*

Antimicrobial Activity of *Andrographis paniculata* AgNP

Figure 37

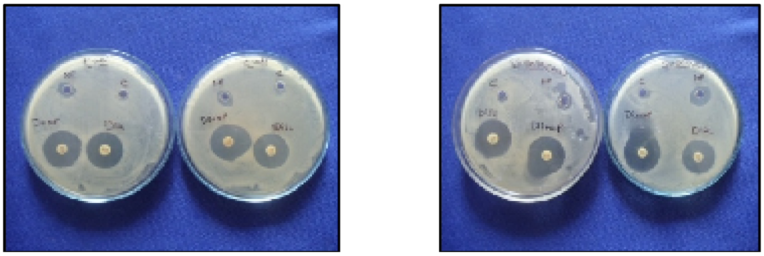
A– *E.coli* **B – *S.enterica***

C– *Sh.dysenteriae*



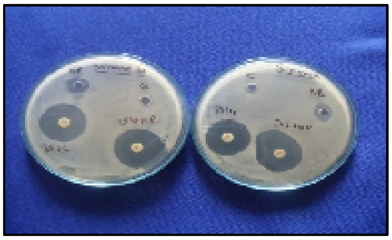
Antimicrobial Activity of *Curcuma longa* AgNP

Figure 38



A– *E.coli* **B – *S.enterica***

C– *Sh.dysenteriae*



Antimicrobial Activity of *Glycyrrhiza glabra* AgNP

Figure 39

The antimicrobial activity of 10, 20, 30 µl silver nanoparticle concentrations delivered in the agar well for the four medicinal plants and control (plant extracts) (Table XV). To start with, the efficacy of antibacterial activity of AgNPs *(Leucas aspera)*at three different concentrations of 10, 20, 30 µl and control was tested in duplicates against the enteropathogenic species. It was found that 30 µl showed a maximum zone of microbial inhibition (of 6mm), compared to the control (of 1mm) against *E.coli (MTCC 40). At* 30 µl, there was8 mm inhibitory zone of against the control of 2 mm against *S.enterica (MTCC 3219). At* 30 µl, the zone of inhibition was 7mm, when compared to the control of 2 mm against *Sh.dysenteriae (PSGIMS&R)* respectively (Figure 36).

By determining the efficacy of antibacterial activity of *Andrographis paniculata* AgNP of 10, 20, 30 µl and control were tested in duplicates against *E.coli (MTCC 40), Salmonella enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R).* It was observed that30µl AgNP (*Andrographis paniculata)* showed maximum zone of microbial inhibition were 7mm and 6mm than the control (plant extracts) of 2mm against both *E.coli (MTCC 40)* and *S.enterica (MTCC 3219)* respectively. Similarly, 30 µl AgNP (*Andrographis paniculata)* showed a maximum zone of inhibition of 10mm, when compared to the control of 3mm, tested against *Sh.dysenteriae (PSGIMS&R) (*Figure 37).

Antimicrobial activity of *Curcuma longa* AgNPat different concentrations of 10, 20, 30 µl and control were tested in duplicates against *E.coli (MTCC 40), S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R).* It was observed that 30 µl showed a maximum zone of inhibition of 8 mm than the control of 1 mm against *E.coli (MTCC 40).* Thezone of inhibition was 10 mm and 12 mm, compared to the control of 2 mm against*S. enteric (MTCC 3219)* and*S. dysenteriae (PSGIMS&R)* respectively(Figure 38).

Antibacterial efficacy of *Glycyrrhiza glabra* AgNPof concentrations of 10, 20, 30 µl and control were tested in duplicates against *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R).* It was observed that 30 µl showed maximum zone of inhibition at 11mm, compared to the control of

3mm against *E.coli (MTCC 40).* The zone of inhibition was 15mm, compared to 3 mm against *Salmonella enterica (MTCC 3219)* and inhibitory zone of 14 mm, compared to the control of 4 mm against *Sh.dysenteriae (PSGIMS&R)* respectively (Figure 39).Thus, it was observed that *Glycyrrhiza glabra* AgNP showed an excellent antibacterial activity with a maximum zone of inhibitionagainst the enteropathogenic species of *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R)* respectively, compared to the control (plant extracts). Further, in order to confirm thehighest antibacterial efficiency of *Glycyrrhiza glabra* AgNP (30 µl) was tested at varied concentration of AgNP from 30 to 125 µl by agar well disc diffusion method.

Sukirtha *et al.,*(2012) describe that silver compounds are highly posionous to microbes like viruses, bacteria and fungus. Prabhu and Poulose (2012) illustrate that the bactericidal activity of silver nanoparticle, could be due to silver inhibiting the cell transduction causing bacterial cell lysis. Arasu *et al.,* (2010) depict that the small size and surface pattern of silver nanoparticles, which would enhance the surface area to volume ratio, thereby improving the antibacterial activity.

(i) Zone of Inhibition

Table XVI showed the effect of varied concentrations of *Glycyrrhiza glabra* AgNPs at varied concentrations from 10 µl to 40 µl and from 50 µl to 125 µl, compared to the control, which was carried out by Agar well disc diffusion in duplicates and were tested against the enteropathogenic species of *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R)* respectively.

TABLE XVI

DIFFERENT CONCENTRATIONS OF *Glycyrrhiza glabra* AgNP AGAINST ENTEROPATHOGENIC SPECIES

Concentration (µl)	***Glycyrrhiza glabra***		
	Zone of Inhibition (mm)		
	E.coli (MTCC40)	***S.enterica (MTCC 3219)***	***Sh.dysenteriae (PSGIMS&R)***
10	4	3	3
20	6	7	8
30	11	15	14
40	7	9	10
Control	3± 0.35	2± 0.21	4± 0.26
50	5	3	6
75	8	5	8
100	6	6	7
125	9	8	9
Control	2± 0.18	2± 0.15	3± 0.22

In Agar Well Disc Diffusion, each agar plate is made into five wells, inoculated with the enteropathogenic species such as *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R).* Each well is loaded with AgNP (*Glycyrrhiza glabra)* of 10 to 40 µl of AgNP and a control (plant extracts). It was observed that the concentration of 30 µl of the plant extract had the maximum inhibition zones of 11 mm, 15 mm and 14 mm respectively against *E.coli (MTCC40, S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R).* Moreover, the concentration of 30 µl showed the maximum antimicrobial activity against diarrheal pathogens.

Galdiero *et al.,* (2011) suggest that the antimicrobial action of silver mainly depends on Ag+ ions would strongly prevent the microbial growth through inactivation of respiratory enzymes and electron transports, thereby mutating the DNA. Several research studies state that the respiratory function of microbial cells are affected by attaching the silver ions to microbial surface, by inhibiting bacterial growth. Sondi (2004) describe that another mechanism is that the silver ions penetrate deeply into the cell surface of the bacteria, damaging the DNA, thereby killing the bacterial cells.

Ramamurthy *et al.,* (2013) state that since the cell membrane of microbes were negatively charged and silver nano particles were positively charged, it was found that these positively charged AgNP accumulate on negatively charged bacterial cell membrane, causing substantial conformational change in the cell membrane leading to cell death. The other mechanism has been proposed by Danilczuk *et al*., (2006) and Kim *et al*., (2007) is that the formation of free radicals subsequently induces microbial damage leading to effective inhibition of microbes. Guzman *et al.,* (2012) shows the AgNP attaches to the cell membrane and penetrate inside the bacteria. These interactions of nanoparticles cause damage to DNA and proteins, because of which Ag^+ binds to functional groups of proteins, leading to the destruction of microbial pathogens. These silver nano particles show efficient antimicrobial property due to their extremely large surface area, which would provide better contact with microorganisms. Shrivastava *et al*., (2007) report that the binding of the nanoparticles to the bacteria depends on the interaction of the surface area available. The smaller nanoparticles having a larger surface area are easily available for its interaction, which poses a stronger bactericidal effect than the larger particles.

Statistical analysis of the *Glycyrrhiza glabra* plant extract without AgNP (control) at different concentrations (Mean ± SD) showed 3± 0.35, 2± 0.21 and 4± 0.26 against *E.coli (MTCC40), S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R)*which exhibit visible bacterial growth within 24 hours of the study. It was observed that these values were statistically not significant. In other agar plates, four wells were diffused with AgNP(*Glycyrrhiza glabra*) of 50 to 125 µl and a control (only plant extracts) onto the inoculated enteropathogenic species. It was also observed that the control (*Glycyrrhiza glabra*) showed that 2± 0.18, 2±0.15 and 3± 0.22 against *E.coli (MTCC40), S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R)*which were found to be statistically not significant.

(ii) Microbial Plate Count

Table XVII reveals the Antibacterial Activity of *Glycyrrhiza glabra* AgNPagainst Enteropathogenic species by Bacterial Count Method.

TABLE XVII

ENUMERATION OF BACTERIAL COUNTS OF SILVER NANOPARTICLE (*Glycyrrhiza glabra)* AGAINST ENTEROPATHOGENIC SPECIES

Silver Nanoparticle Concentration (µl)	*E.coli (MTCC 40)*	*S.enterica (MTCC 3219)*	*S.dysenteriae (PSGIMS & R)*
10	39	50	46
20	31	51	39
30	24	33	28
40	45	57	50
Control	67 ± 3.67	89 ± 5.84	74 ± 3.25
50	71	97	57
75	78	94	62
100	66	86	69
125	61	72	73
Control	92 ± 4.98	107 ± 6.45	89 ± 4.72

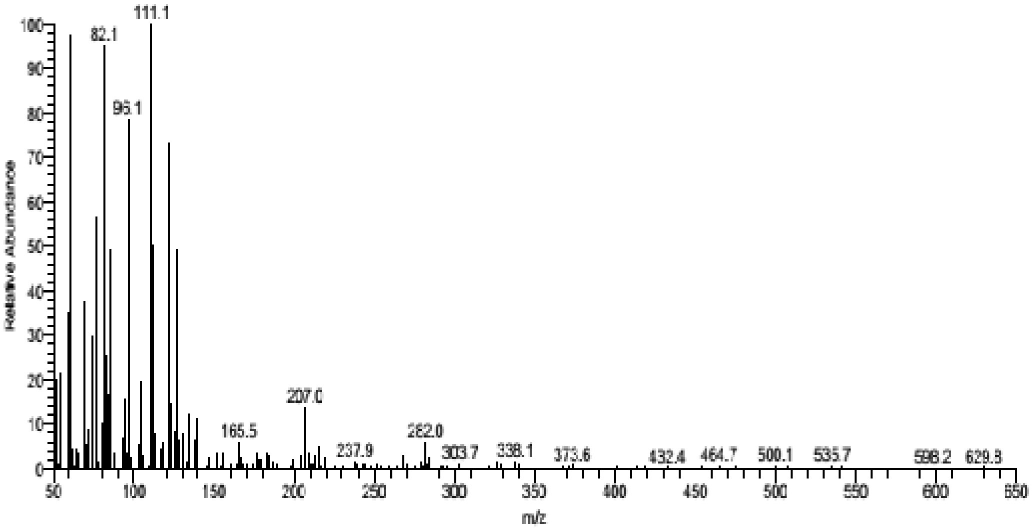
Spread plate method is one of the methods of quantifying the microbes on a solid medium. Table XVII showed that one set of petrid plate (duplicates) consist of five wells loaded with *Glycyrrhiza glabra* AgNPs of 10 to 40 µl along with a control and the other set of petrid plates (duplicates)*Glycyrrhiza glabra* AgNPs were loaded with 50 to 125 µl and a control. Each control is loaded in a petrid plate mainly to avoid the contamination of cultures, to maintain the uniform agar growth medium and uniform swabbing of microbes for each of the agar petrid plates. The control mainly depends on the choice of the growth medium and test microbes, which are being cultured. Each petrid plate of five wells was suspended with *Glycyrrhiza glabra* AgNPs from 10 to 40 µl and control (plant extracts). Table XVII shows that all the control (plant extracts) were tested out independently. The control data were statistically analysed and are expressed in terms of 'mean ± SD' values. 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. In another petrid plates of five wells of *Glycyrrhiza glabra* AgNP loaded from 50 to 125 µl and a control(plant extracts) onto the wells, which are inoculated with enteropathogenic species. The plates are then incubated at 37°C for 24 hrs and then the number of colonies are counted. The control (*Glycyrrhiza glabra*) showed 92 ± 4.98 107 ± 6.45 and 89 ± 4.72 against *E.coli (MTCC40), S. enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R)*. It was observed that these values were statistically not significant .

Among the different concentration of nanoparticle, 30 µl of *Glycyrrhiza glabra* AgNPs 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. Therefore, 30 µl of *Glycyrrhiza glabra* nanoparticles was selected for coating onto commercially available food packages namely PET bottles, Infant Feeding Bottles and Zip Loc covers.

4. Characterization of Plant Extracts with Silver Nanoparticles

a. Chemical Constituents of *Glycyrrhiza glabra*using Gas Chromatography/ Mass Spectrometry (GC/MS)

GC/ MS is a valuable tool for identification of bioactive and pure compounds, which are present at less than 1ng in biological specimens (Liebler *et al.,* 1996). The GC-MS have become a key technological platform for identifying the secondary metabolites present in medicinal plants (Merlin *et al.*, 2009). Figure 40 and Table XVIII depicts the phytocompounds of the *Glycyrrhiza glabra*. The chromatogram of the extracts of *Glycyrrhiza glabra* reveals the five major compounds, which belong to various classes of secondary metabolites.



Gas Chromatography of *Glycyrrhiza glabra*

Figure 40

TABLE XVIII

GC/MS OF *GLYCYRRHIZA GLABRA*

No	**RT**	**Name of the compound**	**Molecular Formula**	**MW**	**Compound Nature**	****Activity**
1	14.75	9-Octadecenal	$C_{18}H_{34}O$	266	Aldehyde	Antimicrobial
2	20.71	Z,Z-2,5-Pentadecadien-1-ol	$C_{15}H_{28}O$	224	Alcoholic	
3	11.58	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	Myristic acid	Antioxidant
4	12.84	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	Palmitic acid	
5	17.34	cis-9,10-Epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	284	Alcoholic	Antimicrobial

The most abundant antioxidant compounds found in *Glycyrrhiza glabra* are the 9Octadecenal (RT=14.75) is an aldehyde compound, cis 9,10 Epoxyoctadecan-1-ol (RT =17.34) and Z,Z,2,5-Pentadecadien-1-ol (RT=20.71) are the major aldehyde andalcoholic compounds which possess the antimicrobial activity. Among the esters and organic acids, the most dominant fatty acids are tetradecanoic acids (RT=11.58), n hexadecanoic acid (RT=12.84) which are capable of scavenging free radicals, due to its antioxidant property. In addition, these phytocompounds of *Glycyrrhiza glabra*may act as a capping and reducing agent in synthesizing the silver nanoparticles.

Bodoprost and Rosemeyer (2007) stated that phytocompounds namely hexadecanoic acid are fatty acid esters, 9,12,15-octadecanoic and trienoic acid are unsaturated fatty acid possessing antimicrobial, antioxidant, hypocholesterolemic, anticancer, anti-arthritic and anticoronary property. In addition, the methyl octadecanoate exhibits antifungal and anti-cancer activities. Saravana (2013) report that there are two alcoholic compounds namely tetradecanoic and dodecanoic acid, which has been used as an antimicrobial and hypocholesterolemic agent.

Santhi *et al*.,(2011) elucidate that *Glycyrrhiza glabra* contains the major phytoconstituents namely aminoacids, fatty acids, nitrogen and esters, among which the most common and dominant organic fatty acids are tetradecanoic acid and hexadecanoic acids*.* It was also stated thatseveral research studies shows that the antioxidants from the medicinal plant sources are the major sources of *Glycyrrhiza glabra,* which areeffective in preventing the oxidation of foods. Friedman *et al*., (2002) describe that several research studies prove that antibacterial activity of *Glycyrrhiza glabra*extracts are highly effective against the food borne pathogens. Kalemba and Kunicka (2003) reveal that major compounds of *Glycyrrhiza glabra* are aldehydes, phenols, ethers, alcohols and ketones, which would act as a natural preservative. Joshi and Singh (1980) report that the phytocompounds namely hexadecanoic acid, 9,12 octadecanoic acid, octadecanoic acid, 9 octadecenoic acid, eicosanoic acid and benzoic acid have been identified in *the G. glabra* root.Haraguchi *et al.,* (2002) described that the phytoconstituents of *Glycyrrhiza glabra* possess highly effective antioxidants and antimicrobial activity. Therefore, due to the presence of phytoconstituents of *Glycyrrhiza glabra*AgNP, which would act as an encapsulating and antibacterial agent.

b. Chemical Constituents by Thin layer chromatography (TLC) (MACHEREY – NAGEL, GERMANY)

Figure 41 shows the presence of Phytoconstituents by Thin Layer Chromatography (TLC)

TLC of *Glycyrrhiza glabra*

Figure 41

Chromatographic Fingerprinting is a very unique tool for the separation and characterization of phytoconstituents. The Phytochemical screening of *Glycyrrhiza glabra* silver nanoparticles by Thin Layer Chromatography (TLC) shows the presence of phenolic compounds with a purple colourasdepicted in Figure 48. The FTIR confirms the presence of phenols, which shows a narrow and sharper peak at 578.06 cm^{-1}which is one of the reasons for its maximum antioxidant potential. Ivanova *et al.,* (2005) report that *Glycyrrhiza glabra*contains the antioxidant compounds viz., phenols which would act against scavenging the free radicals.

c. Functional Groups by Fourier Transform Infra-Red Spectroscopy (FTIR) (SHIMADZU, 8400 S)

FTIR Spectroscopy is a sensitive method for detection of bimolecular composition (Kumar and Prasad, 2011). To determine the functional groups of the synthesized silver nanoparticles from the four chosen medicinal plants, FTIR analysis was performed. The FTIR studies are carried out to investigate the functional groups of the plant extracts, nanoparticles and to identify the correlation between silver precursor salt and protein molecules, which would lead to the stabilization of Silver Nanoparticles. The Control Spectra (Before Reaction with $AgNO_3$) showed a number of peaks reflecting a complex nature of the synthesized silver nanoparticles from all the plant extracts. The band intensities in different regions of the spectrum for the control and test samples (Before and After Reaction with the Silver Nitrate) are analyzed and shown in the Table XIX.

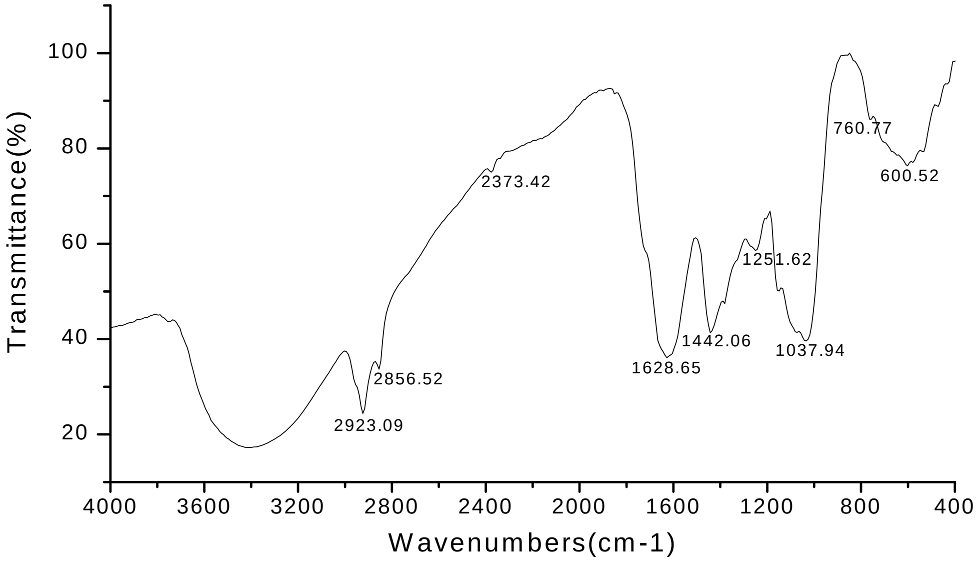
Figure 42 to 45 depicts that the FTIR peaks of the medicinal plant extracts with and without treatment with AgNP.

In the present study, to start with FTIR spectrum of raw extracts (Before Reaction with $AgNO_3$) and AgNP (After Reaction with $AgNO_3$) of *Andrographis paniculata*(Figure 42) showed that the absorption band at 2923.09 cm^{-1} with a shift to 2917.68 cm^{-1} corresponding to aliphatic C-H stretching. In addition, peaks at 2856.52 cm^{-1} also shows a shift to 2849.55 cm^{-1} confirming the presence of aliphatic C-H stretching frequencies. The presence of protein with CH_2 stretching band at 2373.42 cm^{-1} to 2367.22 cm^{-1}, which are observed from raw extracts of *Andrographis paniculata* to the chelated AgNP. The IR band at 1442.06 $cm^{-1}$of raw extracts of *Andrographis paniculata* shows a shift to 1432 cm^{-1} of AgNP, indicating the presence of urea/ starch. A characteristic peak of C=C alkene group stretching of NH amide bending appeared at 1628.65 cm^{-1} to 1608.52 cm^{-1} in both raw and AgNP (*Andrographis paniculata)* indicating the participation of carbonyl group of the carboxylic acid in capping of the AgNP. The FTIR peak observed at600 $cm^{-1}$and 760.77 cm^{-1}(raw extracts) showed a shift to 625.29 cm^{-1}and715.87 cm^{-1}(AgNP) and confirms the presence of alkyl halide, which is attributed to the antibacterial action of nanoparticles.

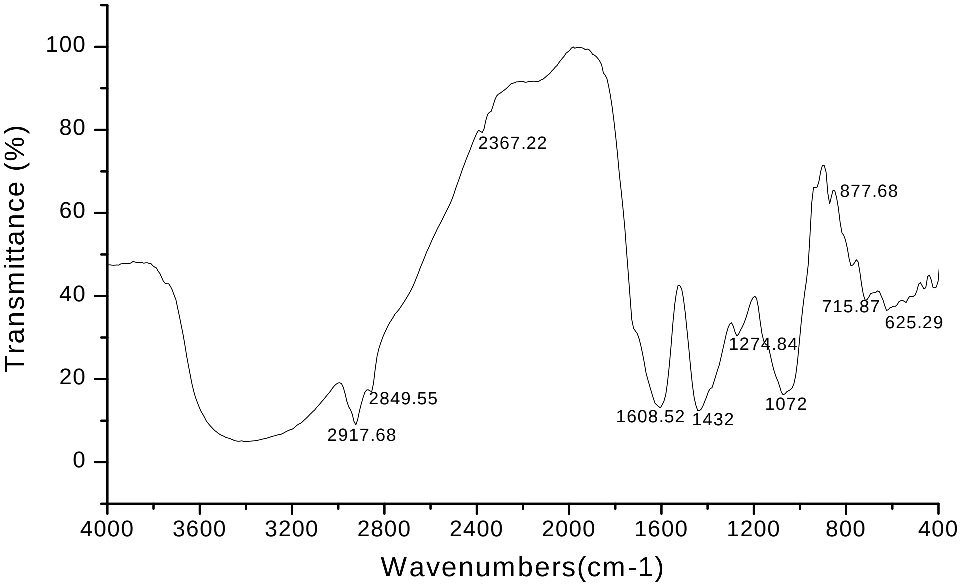
TABLE XIX

FTIR PEAK VALUES OF THE CONTROL (without AgNPs) AND TREATED (with AgNPs) MEDICINAL PLANTS

Medicinal Plants	Without AgNP	With AgNP	Functional Groups
Andrographis paniculata	2923.09	2917.68	Aliphatic C-H stretching
	2856.52	2849.55	Aliphatic C-H stretching
	2373.42	2367.22	Protein/celluloseCH_2 bending second overtone
	1628.65	1608.52	C=C Alkene stretch,NH amide bending
	1442.06	1432	Urea/starch
	1251.62	1274.84	C-N amine stretch
	760.77	715.87	Alkyl halide
	600.00	625.29	Alkyl halide
Leucas aspera	2846.45	2852.65	C-H Alkane stretching
	1635.61	1628.65	C=C Alkene stretch,NH amide bending
	1441.29	1395.61	Urea/starch
	1266.32	1248.51	C-N Amine stretching
	709.67	605.93	Alkyl halide
Glycyrrhiza glabra	3396.90	3410.10	Alcohol OH stretch (H bonded)NH Amine stretch
	2315.35	2371.87	Protein/celluloseCH_2 bending second overtone
	1644.90	1627.87	C=C Alkene stretch,NH amide bending
	1528	1441.29	C=O stretching
	1221.42	1277.94	C-N Amine stretching
	703.52	715.10	Alkyl halide
Curcuma longa	3720.62	3716.65	Alcohol OH stretch (H bonded)NH Amine stretch
	3530.06	3504.52	OH stretch, hydrogen bonded
	3383.74	3390.71	Alcohol OH stretch (H bonded)NH Amine stretch
	2375.74	2378.84	Carboxylic acid,H bonded withOH stretch
	2319.22	2315.35	C-H stretchStarch/cellulose, CH_2 deformation
	1643.35	1637.94	C=C Alkene stretch,NH amide bending
	1521.81	1528.00	NH Amine bending

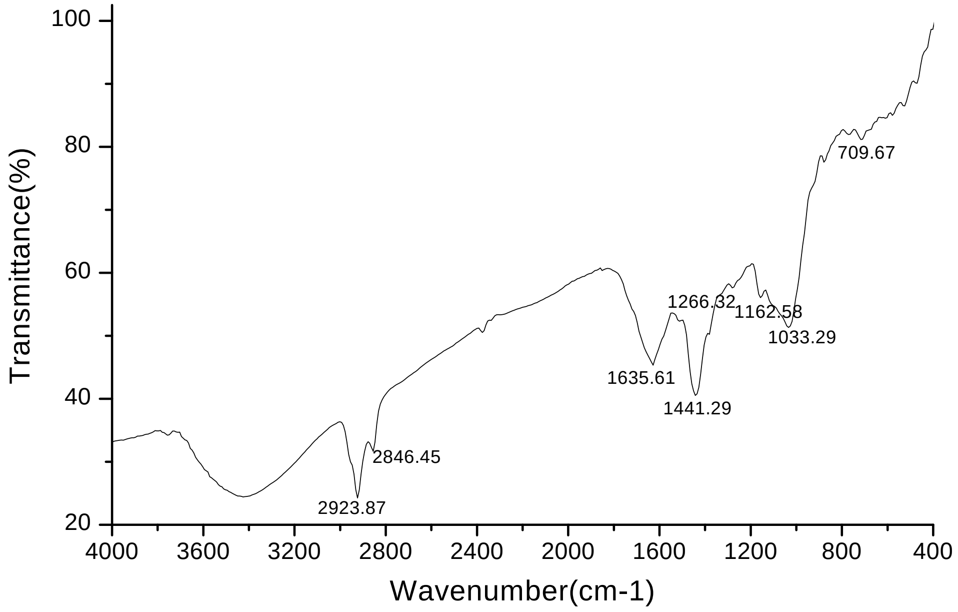


***Andrographis paniculata* (Without AgNP)**

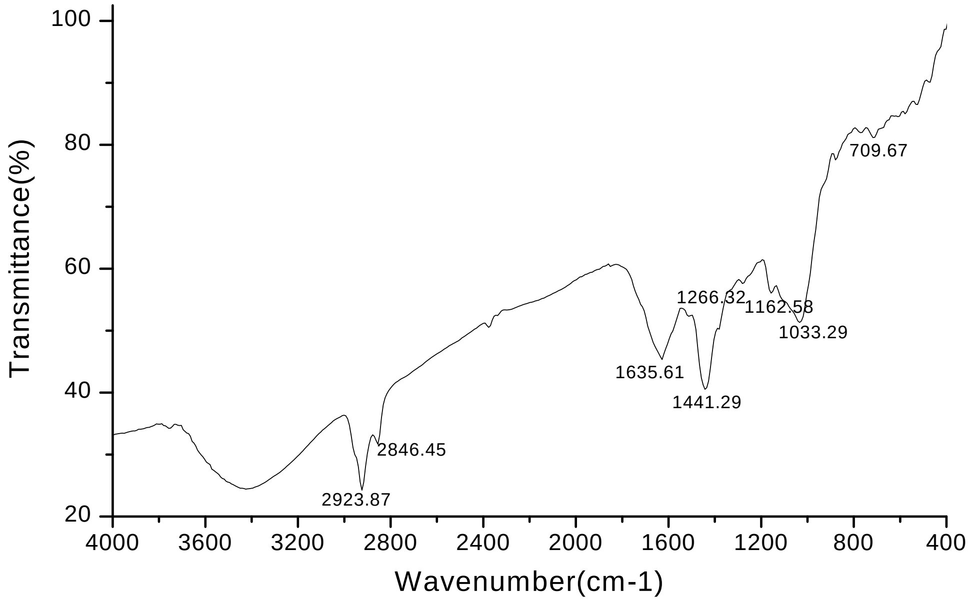


***Andrographis paniculata* (With AgNP)**

Figure 42

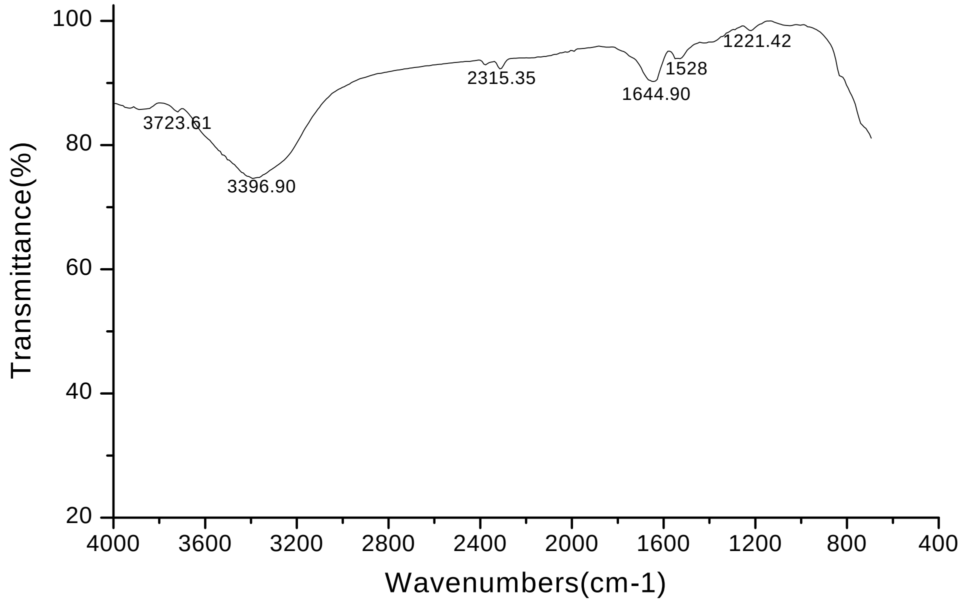


***Leucas aspera* (Without AgNP)**

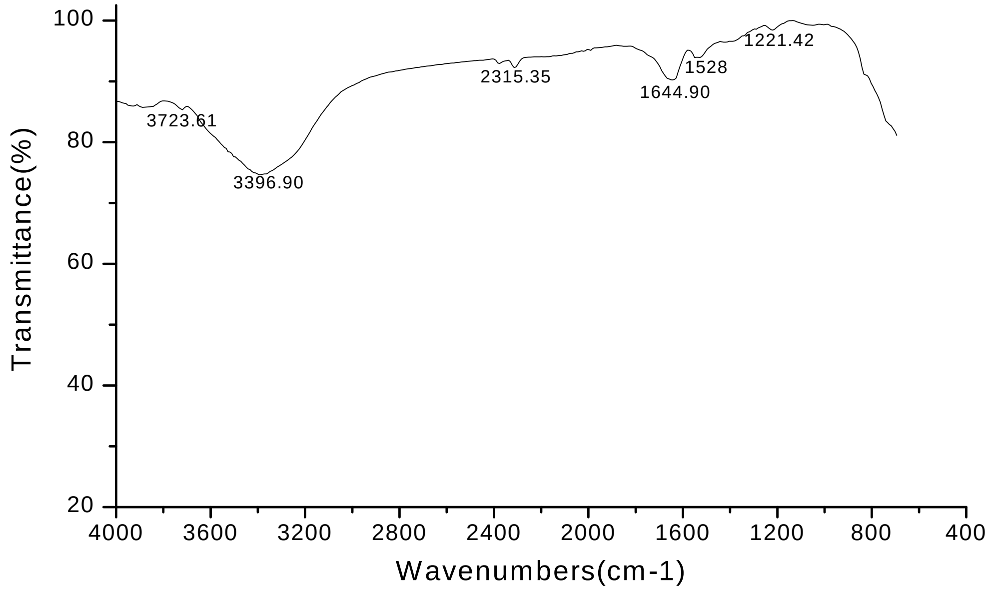


***Leucas aspera* (With AgNP)**

Figure 43

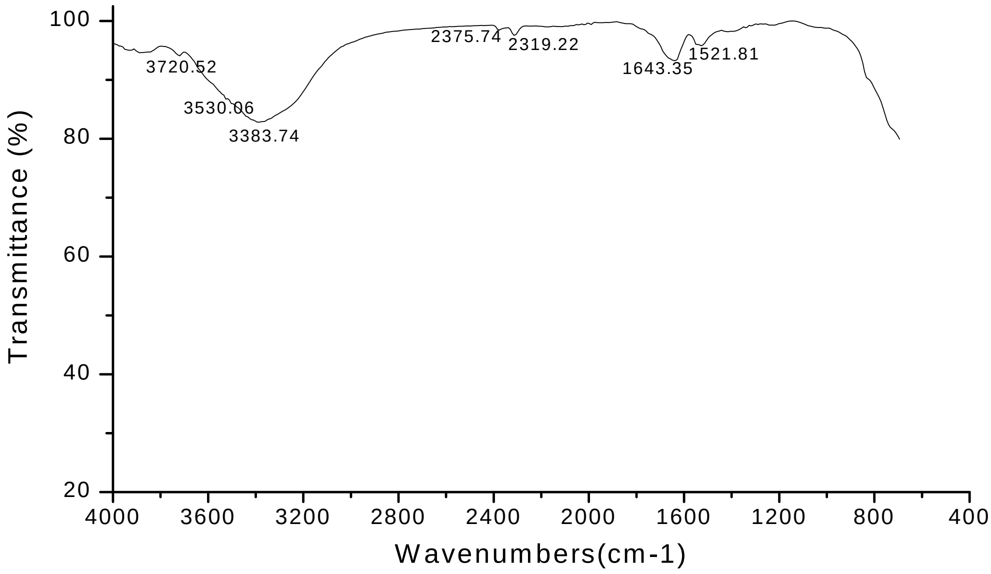


Glycyrrhiza glabra **(Without AgNP)**

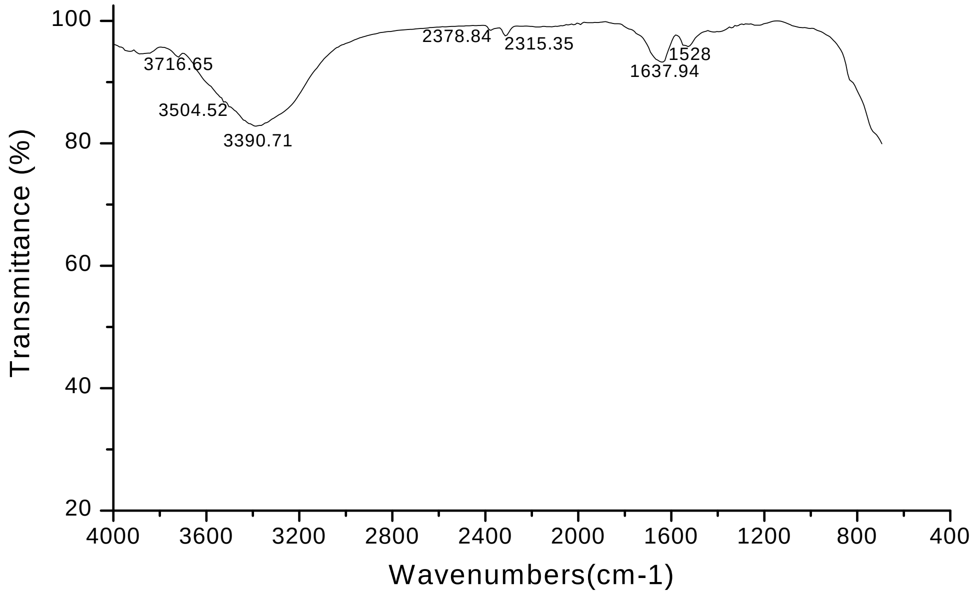


Glycyrrhiza glabra **(With AgNP)**

Figure 44



***Curcuma longa*(Without AgNP)**



***Curcuma longa* (With AgNP)**

Figure 45

This corresponds to the chlorine observed in the Energy Dispersive X ray (EDX) studies forming bond with the silver nanoparticles. Therefore, in the present study, AgNP(*Andrographis paniculata)*is encircled by proteins containing functional groups such as amides, amines, alkanes and alkenes. It was also observed that these functional groups have a stronger capacity with AgNP to provide the highest stability of AgNP.

Secondly, FTIR spectrum of the Raw Extracts (before reaction with $AgNO_3$)and AgNP(after reaction with $AgNO_3$)of *Leucas aspera*(Figure 43)shows the principal peaks at 2846.45 $cm^{-1}$of less intense vibration to 2852.65 cm^{-1} depicting the alkane stretching, 1635.61 cm^{-1} to 1628.65 $cm^{-1}$with the amide bending for C=C alkene stretching. The absorption peak of raw extracts and AgNP (*Leucas aspera)* represent the presence of urea/starch. In addition, the peak formed at 1266.32 cm^{-1} and 1248.51 cm^{-1} confirms the C-N amine stretching. A characteristic peak was observed at 709.67 cm^{-1} and 605.93 cm^{-1} indicating the presence of alky halide groups respectively, which pose the inhibition of microbes.

From the Table XIX and Figure 44 depicts the FTIR spectra of the third plant, *Curcuma longa* of the raw (before reaction with $AgNO_3$)and AgNP (after reaction with $AgNO_3$)shows that the peak at 3720.62 cm^{-1} with a shift to 3716.65 cm^{-1} confirming the presence of alcohol OH stretching with NH amine, 3530.06 cm^{-1} to 3504.52 cm^{-1} with OH stretching of H bonding. Similarly, the peak formed at 2375.74 cm^{-1} and 2378.84 cm^{-1} confirms the carboxylic acid of H bonded with OH stretching. A characteristic peak has been observed at 1643.35 and 1637.94 cm^{-1} indicating the presence of C=C alkene stretch with NH amide bending.

Table XIX and Figure 45 shows that the FTIR spectra of *Glycyrrhiza glabra* raw extracts (before reaction with $AgNO_3$)and AgNP (after reaction with $AgNO_3$)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. It has been observed that FTIR of *Glycyrrhiza glabra* root and colloids shows the functional peak such as C=C, C-O-C, C=O, C-O and C-N corresponds to biomolecules like phenols, flavanoids and terpenoids, which are involved in reduction and capping of silver ions leading to effective chelation of silver nanoparticles. The 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.

Khan *et al.,* (2011) indicated that the presence of amide usually exhibits its vibration peaks from 1500 to 1650 cm^{-1}. Gopinath *et al.,* (2013) state the amide of protein, could bind to AgNP, thereby stabilizing them. The presence of wave number related to amides and amines confirms the protein. Similarly the characteristic peak of *Glycyrrhiza glabra*observed at 1441.29 cm^{-1} confirms the NH amine bending. The band of C-N amine stretching groups of raw and AgNP (*Glycyrrhiza glabra)* at 1221.42 cm^{-1} shows a shift to 1277.94 cm^{-1}. Theivasanthi *et al.,* (2011) stated that proteins could bind with AgNP through free amine groups of proteins. Therefore, AgNP stabilization could be possible by surface bound proteins. Joglekar *et al.,* (2011) describe that these protein could encapsulate AgNP, thereby acting as a capping agent. From several literature studies, it was confirmed that NH/CO groups would bind with the AgNP, in which the proteins and peptides possess a stronger affinity to bind with the metals. Therefore, these proteins act as an encapsulating agent, which forms a layer covering the metal nanoparticles. It was also illustrated that these layers may prevent the agglomeration, thereby stabilizing the nanoparticle in aqeous medium.

Thiols and polyphenols are the two major phytocomponents that induce antioxidant potential. *Glycyrrhiza glabra AgNP* shows astronger and a narrow peak at 578.06 cm^{-1} corresponding to phenol possessing a maximum antioxidant potential, while the same peak is weak in *Leucas aspera* corresponding to its poor antioxidant potential. Therefore, high antioxidant activity of *Glycyrrhiza glabra* may be attributed to phenols.A peak observed at 702.65 cm^{-1} and 715.87 cm^{-1} confirms the presence of alky halide contributes for its antimicrobial activity. Shankar *et al*. (2009) report that the antibacterial property of *Glycyrrhiza glabra* nanoparticles are due to the presence of phytocompounds such as phenols, alcohols, alkanes, alkenes and alkyl haides, which are based on the presence of functional group by FTIR. Scalbert (1991) states that the diarrheal disease areon the increase globally and its treatment by synthetic drugs are linked up with side effects. Therefore, utilizing the active (phytochemical) compounds from the plant extracts are safer and non toxic and contribute to the antibacterial properties to solve diarrheal problems. Xiao (2014) describes that the flavonoids are the major phytoconstituents present in these four medicinal plants, which are used for inhibiting the microbes of diarrheal illness. Dharmananda (2003) illustrate that tannins are astringent phytocompounds in plants and which are used in curing stomach disorders such as dysentery and diarrhea.

Panacek *et al.,* (2006) report that gram positive and negative bacteria, differ in the structure of cell walls. The cell wall of gram negative bacteria are composed of a thin peptidoglycan layer and lipopolysaccharide layer, made of covalently linked polysaccharides and lipids with a negative charge providing weak permeability to AgNPs with positive charge. The presence of thin peptidoglycan layer makes it easy for the AgNPs to bind with the gram negative bacterial cell wall. Amro *et al.,* (2000) propose that several research studies suggest that the antimicrobial effect of AgNPs are due to the formation of pits, thereby causing damage to the bacterial cell wall. The morphological changes of gram negative bacterial cell wall causes an increase in permeability of the cell membranes allowing AgNPs to penetrate into the cell, thereby, breaking the double stranded DNA, resulting in cell death.

Ahmed *et al.,* (2011) mention that the antimicrobial effect of silver nanoparticles could be due to the stability of the suspension that changes the phosphotyrosine structure of the microbial proteins, thereby inhibiting its growth. In addition, silver nanoparticles from the plant extracts are highly effective against gram negative bacteria, due to the presence of peptidoglycans possessing the lipoteichoic / teichoic acids with negative charges. These charges may attract and bind with more positive charges of colloidal silver ions. Hence, gram negative bacteria may allow more silver ions to reach the cytoplasmic membrane of cell, thereby damaging the microbial cells. Warsnoicharoen *et al.,* (2011) found that silver nanoparticles have an ability to interfere with metabolic pathways.

Sereemaspun *et al.,* (2008) describe that the inhibition of microbial are caused by the diffusion of Silver Nanoparticles across the microsomal membrane. Among the four FTIR spectraof medicinal plants, *Glycyrrhiza glabra* confirmed the presence of alkyl halide as a strong and a potent antimicrobial agent. The peak at 1625.82 and 668.05 cm^{-1}corresponds to Ag-O stretching and deformation vibration respectively. The peak of Ag- CHO bond is at 2830 – 2695 cm^{-1}.The stretching vibration peak of Ag-OH is at 3300 - 3600 cm^{-1}.

d. Constituent Elements from Energy Dispersive Auger X Ray analysis (EDX OXFORD INCA)

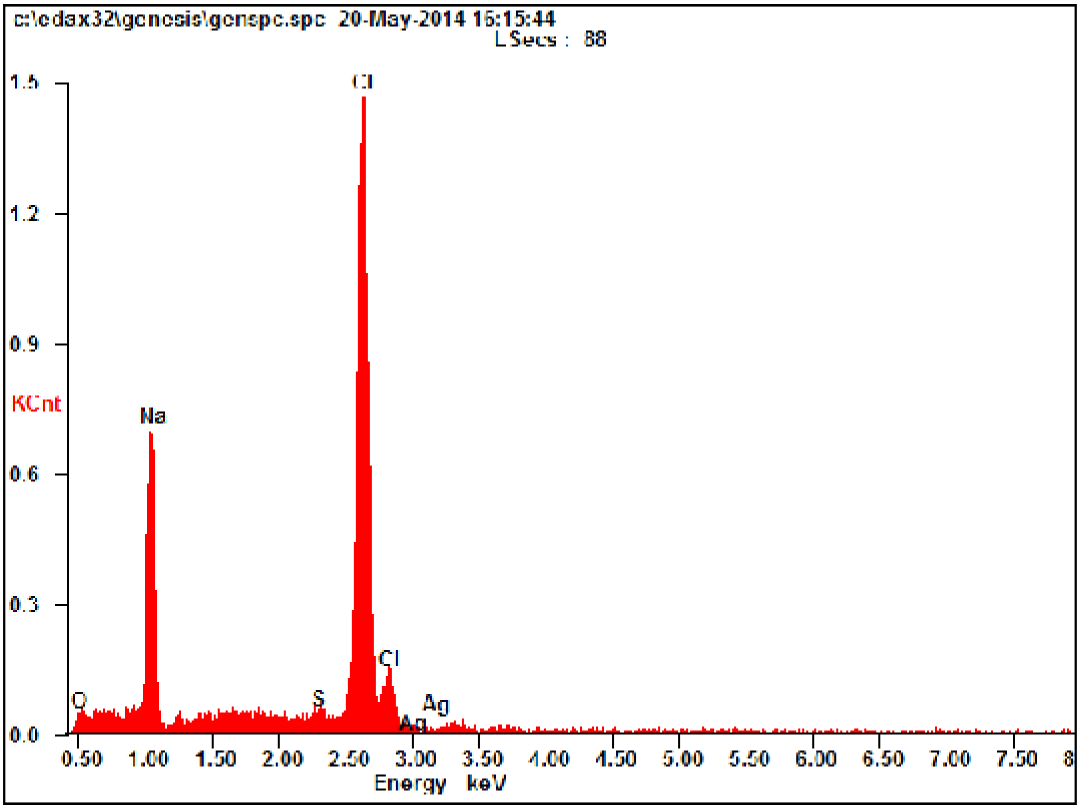
Table XX depicts the EDX of *Glycyrrhiza glabra* AgNP

TABLE XX

EDX OF *Glycyrrhiza glabra* AgNP

Element	Wt %	At %
O K	11.93	19.97
NaK	35.85	41.75
S K	02.22	01.86
ClK	47.39	35.78
AgL	02.60	00.64

The elemental analysis of *Glycyrrhiza glabra*indicated several inorganic elements with few cationic namely Na, Ag and few anionic namelyCl and S in the EDX studies and are presented in Table XX (EDX). The silver content was about 0.64 Atomic weight % (Figure 46) which acts as a potent chelating and microbial inhibiting agent, along with the phytochemicals of the plant extract. The highest level of cationic impurity is observed in *Glycyrrhiza glabra* silver nanoparticleswith impurity such as Sodium *of* 41.75 per cent.



EDX of *Glycyrrhiza glabra* AgNP

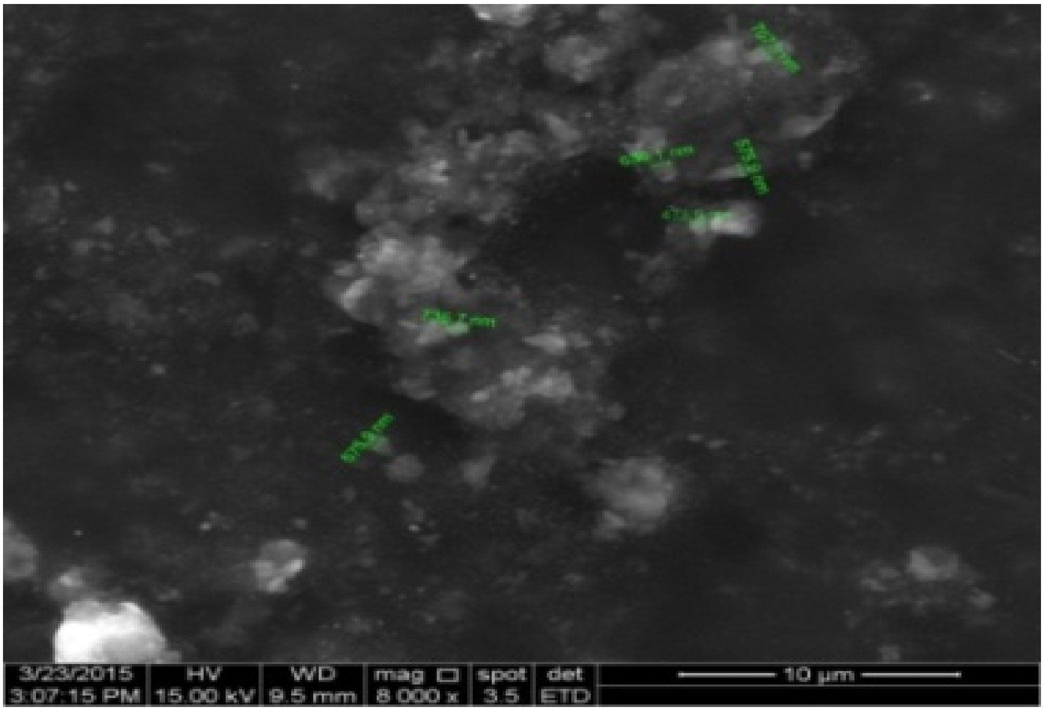
Figure 46

In addition, higher percentage of anionic impurity namely Chlorine and Sulphur of 35.78 per cent and 1.86 per cent respectively was observed. The higher percentage of impurities of Na and Cl of *Glycyrrhiza glabra* are due to the soil rich in minerals, which may vary based on its variedgeographical location. The FTIR peak observed at 600 cm^{-}1 and 760.77 cm^{-1}, (raw extracts) shows a shift from 625.29 cm^{-1} to 715.87 cm^{-1}, (AgNP) and confirms the presence of alkyl halide, contributing to the antibacterial action of nanoparticles. This corresponds to the chlorine observed in the EDX forming bond with silver nanoparticles.

e. Morphology of *Glycyrrhiza glabra* Silver Nanoparticles using Scanning Electron Microscopy (SEM)

Figure 47 depicts the morphology of *Glycyrrhiza glabra* Silver Nanoparticles using Scanning Electron Microscopy (SEM**).**

617.57 nm



SEM (Magnification X 20,000) of Biosynthesized AgNP using *Glycyrrhiza glabra*

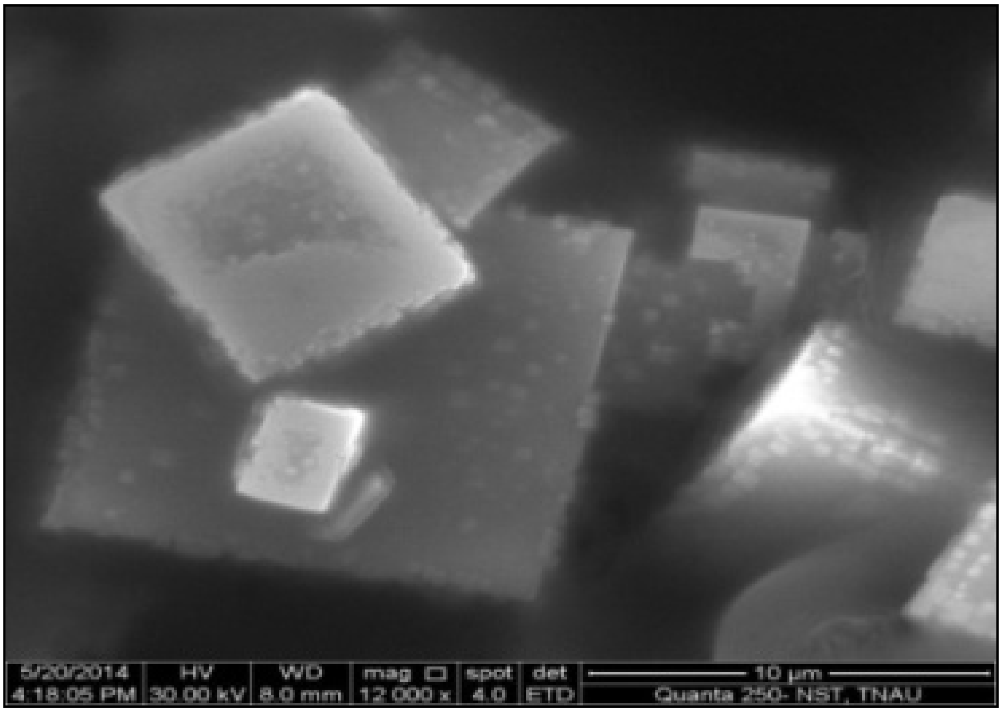
Figure 47

SEM of *Glycyrrhiza glabra* Nanoparticles shows spherical shaped nanoparticles homogenously distributed with an average grain size of 617.57 nm (Figure 47) as observed from the greatest magnification (X 20,000). Only a few studies have been reported on SEM of *Glycyrrhiza glabra* nanoparticles. Dinesh *et al.,* (2012) state that the SEM of *Glycyrrhiza glabra* Silver Nanoparticle are of size 20 – 30 nm, well-dispersed and spherical in shape.

f. Structure and Crystallinity of Transmission ElectronMicroscopy (TEM) (HITACHI S 4700)

Figure 48 depicts the Structure and Crystallinity of Transmission Electron Microscopy (TEM) (HITACHI S 4700).

304.68 nm



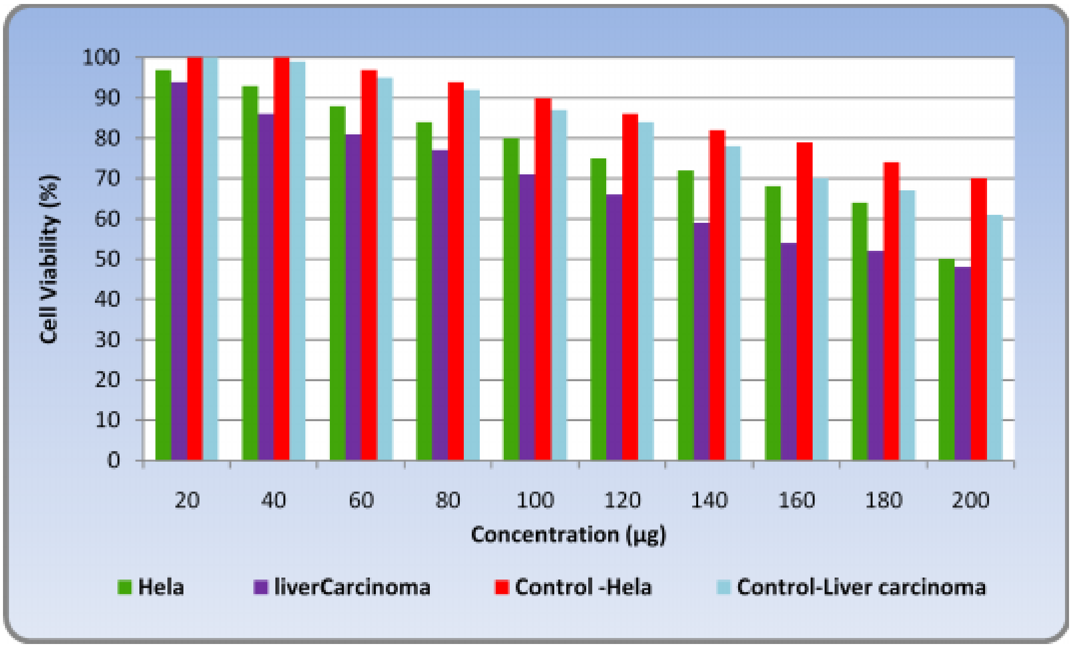
TEM (Magnification X 20,000) of Biosynthesized Silver Nanoparticles using *Glycyrrhiza glabra*

Figure 48

The TEM of synthesized *Glycyrrhiza glabra* silver nanoparticles shows polydispersed colloidal particles with average grain size of 304.68 nm (Figure 48). The high resolution TEM imagesindicates the good crystallinity of the nanoparticles.Huang *et al.,* (2007) described that the TEM image of *Glycyrrhiza glabra* AgNPs indicate good crystallinity of the nanoparticles, in which shape and size of the silver nanoparticles are controlled by adjusting the reaction mixture.

g. Anticancerous Activity by MTT and Gel Electrophoresis

Figure 49 depicts the Anticancerous Activity by MTT and Gel Electrophoresis.

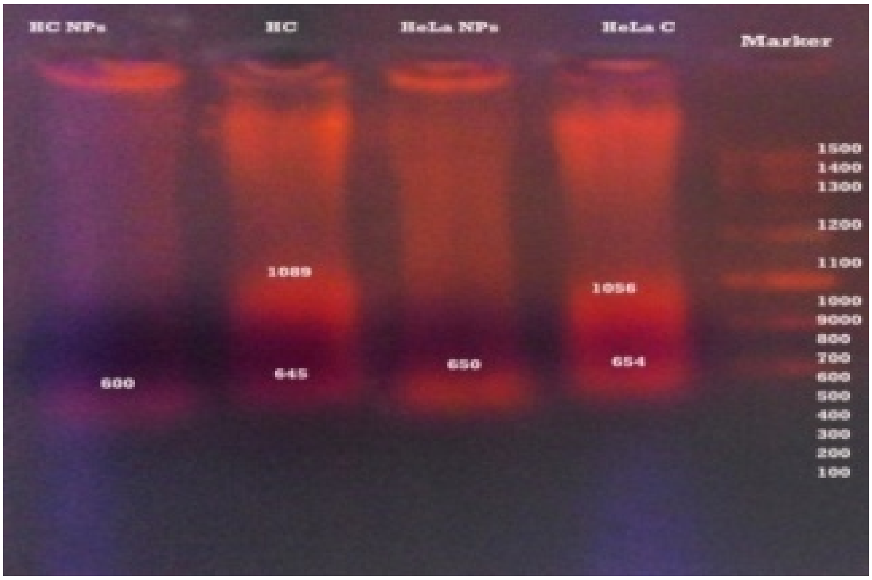


MTT of Silver Nanoparticles *(Glycyrrhiza glabra)* against HeLa and Liver Carcinoma Cell Lines

Figure 49

Cell Viability determination was made using MTT assay which was studied for *Glycyrrhiza glabra* causing a significant cytotoxicity. From the analysis, it was found that 200 µl of silver nanoparticles showed 50 percent apoptosis in HeLacell line and 48 percent in human carcinoma apoptosis at a concentration of 200µl compared to control. The apoptosis of cancer cells could be the possible mechanism induced by the cytotoxic effect of biosynthesized silver nanoparticles from *Glycyrrhiza glabra.* In addition to antimicrobial property*, Glycyrrhiza glabra* possesses the anticancer activity.

Figure 50 shows the Gel Electrophoretic pattern of *Glycyrrhiza glabra* against the HeLaand liver carcinoma cell lines



Gel Electrophoresis against HeLaand Liver Carcinoma Cells

Figure 50

The silver nanoparticles (*Glycyrrhiza glabra)* has led to a longer tail DNA damage with HeLaand liver carcinoma cell lines compared with control and marker. It was observed that the DNA gel electrophoresis of DNA fragmentation was enhanced with increasing exposure to silver nanoparticles *(Glycyrrhiza glabra).*

Phase II

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

Food borne diarrheal disorders posea significant health risk worldwide.According to the reports of the Integrated Disease Surveillance Project of India, India has the highest diarrheal outbreaks, which are more than the double as 120 diarrheal outbreaks as seen in 2009, compared to 50 diarrheal outbreaks in 2008. Diarrheal diseasesare caused by microbial contamination of enteropathogenic species such as *E.coli, S.enterica* and *Sh.dysenteriae* respectively, which are specifically correlated to the food borne diarrheal illness and are becoming a major health burden, leading to high morbidity and mortality. The global burden of infectious diarrhea affecting 3 -5 billion people annually, especially affecting young children is caused by contamination of foods. Thefood borne diarrheal illness are caused by consuming foods or liquids contaminated with bacteria. These pathogens cause diarrheal illnessby invading and multiplying in the intestinal tract and lining of intestine by releasing toxins. The severity of food borne diarrheal illnesses are largely dependent on the infective dose of the pathogen.

In tropical countries like India, most of the unprocessed and processed foods are stored at warm temperature in plastic containers such as PET (Poly Ethylene Terephthalate) and Zip loc covers (Poly Ethylene) for their convenience. Infant feeding bottles made of (Poly Propylene) are widely used to feed the milk/juices at room temperature to infants. At warm temperatures, there is the highest multiplication of microbes that are the main causative factors of food borne illnesses such as diarrhea. Use of very less amount of nanoparticles offers a great potential to food packages by improving the thermal, barrier properties and shelf life of foods (Blaser *et al.,* 2008). Nanoparticles coated onto the surface of the packaging materials cause the electrostatic force of interaction between food packaging material and the nanoparticles and this ensuresthe stability of the nanocoating onto food packages.

Hence in the present study, silver nanoparticles were selected based on their antioxidant and antimicrobial property and the resistance against diarrheal pathogens. The study aims to evaluate the shelf life of foods namely the tomato puree and lemon juice stored in nanocoated food packages of PET and Zip loc covers. In addition, the shelf life of milk and lemon juice are also evaluated on nanocoated feeding bottles against the diarrheal pathogens of silver nanoparticles synthesized from the four medicinal plants. *Glycyrrhiza glabra* Ag nanoparticles showed smaller particle size with a highly stable zeta potential. In addition, it also contains the highest antioxidant and antimicrobial property against the enteropathogenic species. Therefore, antibacterial activity of *Glycyrrhiza glabra* AgNPs was tested at varied concentrations of Ag nanoparticles from 10 to 40 µland 50 to 125 µl with the commercially available food package materials like PET, zip loc covers and infant feeding bottles (PP) for enumerating against the diarrheal species and the shelf life of foods are discussed below'.

1. Antimicrobial Property and Shelf Life of Nanocoated Food Packages

a. PET (Poly Ethylene Terephthalate)

The following specifications of PET material were used for the present study :

PET bottles (150 ml)

Material – Poly Ethylene Terephthalate (PET)
Neck size – 25 mm
Weight – 12.5 gms
Colour – transparent white colour with cream cap
Shape – cylindrical

PET is one of the most important packaging materials and are extensively used as a storage containers for storing the food. These PET materials occur in varied forms which possess properties such as strength, high heat resistance, chemically inertness, flexibility, toughness, burst strength and are inexpensive. In addition, these PET materials have a low permeability to water vapour, high permeability to oxygen, carbon dioxide and other gases. Due to the flexible properties of PET, they are widely used in storing a wide range ofvaried pH food products. Hence, in the present study, to solve the diarrheal illness occurring through contaminated foods, these food packaging materials were coated with the *Glycyrrhiza glabra*silver nanoparticles at varied concentration and tested for shelf-life of foods.

Table XXI and Figure 51 depicts the ANOVA (analysis of variance) for the antimicrobial activity of varied concentration of *Glycyrrhiza glabra* nanoparticles with PET materials, control (before coating) and experimental (after coating) with AgNP against *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *S.dysenteriae (PSGIMS&R)*that are at one percent level of significance.

TABLE XXI

ANOVA of ANTIMICROBIAL ACTIVITY OF *Glycyrrhiza glabra* NANOPARTICLE WITH PET MATERIAL AGAINST *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R)*

Glycyrrhiza glabra **Nanoparticle concentration (µl)**	***E.coli (MTCC 40)***		***S.enterica (MTCC 3219)***		***Sh.dysenteriae (PSGIMSR)***	
	Exp	**Percentage (%)**	**Exp**	**Percentage (%)**	**Exp**	**Percentage (%)**
10	36	35.71	80	11.11	55	23.61
20	28	50.00	74	17.78	50	30.55
30	23	58.93	48	46.67	42	41.67
40	37	33.93	66	26.67	49	31.94
Control	56	NIL	90	NIL	71	NIL
50	54	15.63	72	22.58	61	19.74
75	52	18.75	81	12.9	63	17.1
100	47	26.56	83	10.75	58	23.62
125	41	35.94	87	6.45	50	34.21
Control	64	NIL	93	NIL	76	NIL
S Ed	2.07		4.00		3.21	
CD	5.88**		11.38**		9.15**	

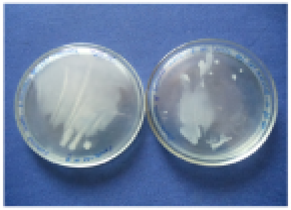
** Significant at (P< 0.01) level; SE – Standard Error; CD- Critical difference; Exp – Experiment; Cont – Control

To the best of our knowledge, not of much research work has been done specifically on antibacterial property of Glycyrrhiza glabra AgNP along with commercially available food material as a nanocoating onto food packages.

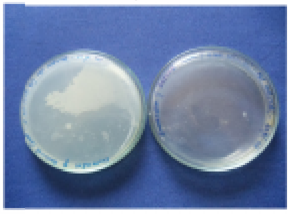
In the present study, phase I reveals that among the varied concentrations, 30 µl of *Glycyrrhiza glabra* AgNP has a smaller particle size, stable zeta potential with highest antioxidant and antimicrobial potential and hence is selected as the best colloidal solution for coating onto the food packaging material such as PET, infant feeding bottles (Poly Propylene) and Zip lock covers (Poly Ethylene).

By spread plate method, one set of sterile petrid plate (duplicates) of experimental group (nanocoated) *Glycyrrhiza glabra* AgNPs along with the sterile PET material of 10 to 40 µl and control (only PET material) and other set of sterile petrid plates (duplicates) of *Glycyrrhiza glabra* AgNPs of 50 to 125 µl were tested along with a control(only PET material). Control was maintained for each set of petrid plates mainly to avoid the contamination of microbial cultures and media in the environment, thereby maintaining a uniform agar growth media and swabbing of microbes for each of the agar petrid plates. The control (uncoated) mainly depended on the choice of the growth medium and test microbes, which were being cultured. A known concentration of *Glycyrrhiza glabra* AgNP from 10 to 40 µl, 50 to 125 µl along with the PET material and a control (only PET material) were swabbed and spread all over the surface of the agar plate, using a sterile glass spreader. The petrid plates were then incubated at 37 °C for 24 hours and then the number of microbial colonies are counted. Among the varied concentration of Ag nanoparticles, it was observed that 30 µl *Glycyrrhiza glabra* AgNP had the best antimicrobial activity and 50 µl had the least antimicrobial activity (Figure 51).

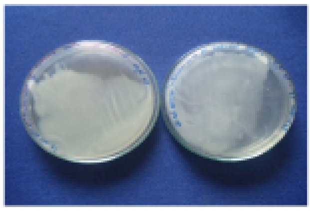
***E.coli*(MTCC 40) - PET materials**



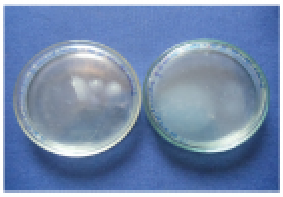
10 µl and 20 µl of NP



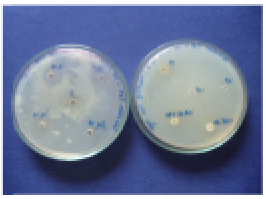
30 µl and 40 µl of NP



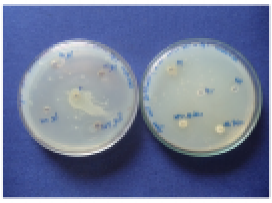
50 µl and 75 µl of NP



100 µl & 125 µl NP



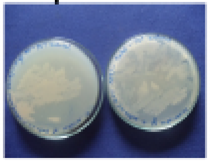
10, 20, 30 & 40 µl NP



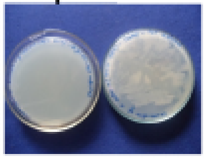
50, 75, 100 & 125 µl NP
Control

***S.enterica(MTCC 3219)* - PET materials**

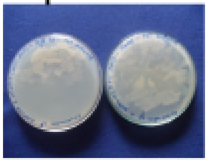
10 µl & Control



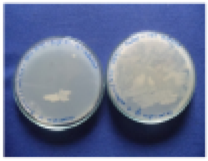
20 µl & Control

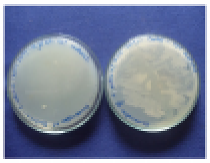


30 µl & Control

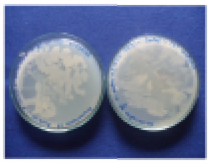


40µl &Control

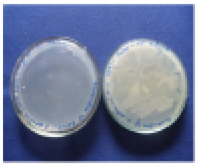




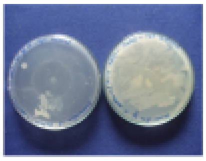
50µl & Control



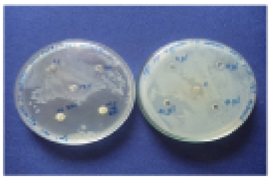
75 µl & Control



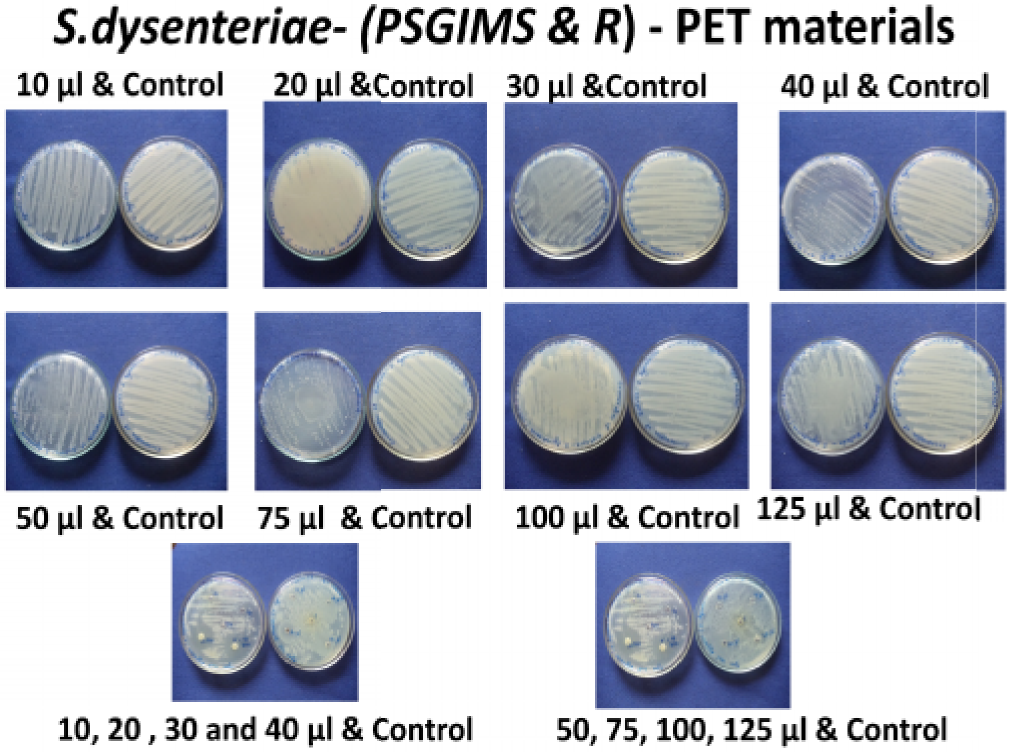
100 µl & Control



125 µl & Control



10, 20, 30, 40 µl & Control



Antimicrobial Activity of *Glycyrrhiza glabra* Nanoparticles with PET materials against *E.coli (MTCC 40), S.enterica (MTCC 3219) and Sh.dysenteriae(PSGIMS& R)*

Figure 51

It was observed that 30 µl of experimental group (nanocoated) of AgNP (*Glycyrrhiza glabra)* showed 58.93 per cent of maximum inhibitory activity ($P< 0.01$) against *E.coli (MTCC 40),* compared to the control (uncoated) which exhibited enormous growth of *E.coli (MTCC 40)*.

Statistical analysis between the experimental (nanocoated) and control group (uncoated) PET bottles shows the good antimicrobial activity of *Glycyrrhiza glabra* AgNP with the PET against the *S.enterica (MTCC 3219).* It was observed that among the varied concentration of AgNP, 30 µl showed the highest inhibition of 46.67 per cent inhibition against *S.enterica (MTCC 3219)* significant at ($P<0.01$). At all concentrations of AgNP, samples was statistically significantly ($P< 0.01$) with higher antimicrobial activity over control samples.

The experiment (nanocoated) PET bottles of *Glycyrrhiza glabra* AgNP showed that 30 µl had 41.67 per cent i.e., highest microbial inhibition, compared to the control (uncoated) PET bottles which showed higher growth of *S.dysenteriae (PSGIMS&R).* Statistical analysis was done between the experimental (nanocoated) and control (uncoated) PET bottles. The difference between these values werefound to be significant at one percent. Therefore, 30 µl of *Glycyrrhiza glabra* AgNP was found to be the best colloidal suspension possessing the highest antimicrobial synergistic effect along with the PET material and hence, 30 µl of *Glycyrrhiza glabra* AgNP was selected as a nanocoating onto the PET bottles and then tested for its shelf life.

As discussed earlier from phase I, Table XVIII showed that the Ag nanoparticles (*Glycyrrhiza glabra)* possess the highest antioxidants as seen from GC/MS which are Octadecenal (RT=14.75) an aldehyde compound, cis 9,10 Epoxyoctadecan-1-ol (RT =17.34) and Z,Z,2,5-Pentadecadien-1-ol (RT=20.71), two major alcoholic compounds possessing antimicrobial property. In addition, the most dominant fatty acids namely tetradecanoic acids (RT=11.58), n hexadecanoic acid (RT=12.84) are also present that have antioxidant property of scavenging the free radicals. The nanocoating of Ag nanoparticles are rich in antioxidants, when coated onto the food packages acts as a self cleansing antimicrobial agent in food packages, thereby prevent the oxidation of foods, enhancing their shelf life. Garland (2004) stated that Ag nanoparticles with higher stability interacts with enzymes and protein, inducing oxidative stress, destruction of mitochondria, thus causing the apoptosis of bacterial cell. The nanoparticles penetrate deeply more efficiently into bacterial cells producing enhanced antibacterial effect.

The high surface area to volume ratio causes a higher reactivity with bacterial cell and inability to cross the bacterial membrane. It thus becomes toxic to the cell wall of gram negative bacteria, thereby inhibiting the diarrheal species. These Ag nanoparticles (*Glycyrrhiza glabra)* when incorporated as a nanocoating in commercial food packages acts well as a antimicrobial agent, which may tend to increase shelf life, thereby keeping the food safe for human consumption. European Union (EU) has established the regulations for nanoparticles as a food ingredient incorporated as a coating/film as a novel food regulation (EC, 2009) 258/97.The U.S., Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled water and in the EU standard, silver is accepted under directive 94/36/EC as a colouring agent (E-174) with no restrictions. According to European Food Safety Authority (EFSA), the silver nanoparticles impregnated in food packages as nanocoatings are used in various processed foods. The silver content in these nanocoated packages should be upto a maximum of three per cent. In the present study, nanocoated food packages, only silver is used and regulated with a maximum limit of 0.017mg/kg in foodstuffs and 1 mg/kg for drinking water (FDA/CFSAN). As far as nanosilver is concerned, the colloidal solutions are accepted in the U.S and commercialized as nutrition supplements, which are claimed to serve as a beneficial effects on human health. Additionally, the EFSA has proven that the silver zeolites, silver zirconium phosphates, and silver coated onto glasses and food packages, which would act as anadditive for food contact materials with a general restriction of ≤0.05 mg/kg of silver onto the food is acceptable. Regardless of the stringent regulations, silver still remains the most widely used antimicrobial polymer additive in food applications.

The silver nanoparticles are widely used as nanocoatings onto the surfaces of the food polymers. The novel properties of silver nanoparticles are due to their unique chemical, magnetic, electronic, thermal and optical properties, which are currently being utilized in food packaging industry. Hence, in the present study, the methods of nanocoating onto PET, PP and zip loc polymer had the rotating coating and formsthe oxygen-diffusion barriers for the plastic material. These nanocoatings are found to be efficient as they prevent oxidation and retain carbon dioxide and act as oxygen scavengers. Due to antioxidant and antimicrobial property of AgNP (*Glycyrrhiza glabra)*they act as self cleansing agents in food packages and prevent the oxidation. Thus they inhibit the bacterial growth and increase the shelf life of foods. The enhanced antibacterial effect of nanosilver are due to higher stability of the nanoparticles that penetrate more efficiently inside the bacterial cells, when compared to silver ions.Sekhon (2010) illustrated that the silver nanoparticles are used as a coating material onto plastic containers. Due to its high reactivity it has the ability to cross the bacterial membrane barriers, thereby becoming toxic to the bacterial cell wall. It is also observed thatthese silver nanoparticles would also act as a novel antimicrobial coating agent in food packages.

i. Shelf Life of Tomato puree in Nanocoated PET bottles

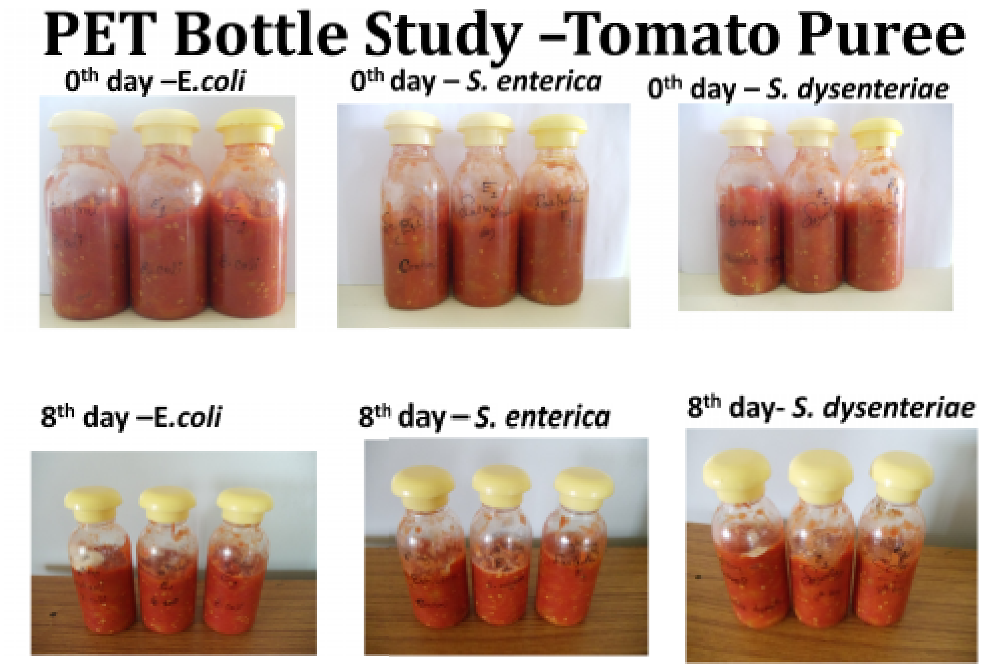
To determine the efficiency of these nanocoated PET bottles its shelf-life study of acidic foods such as tomato puree and lemon juice were also studied.

Tomato *(Lycopersicon esculentum)* is a vegetable grown throughout in tropical and temperate regions of the world. It is an important herbaceous perrenial grown for its edible fruit as an annual vegetable in temperate regions. It has the ability to raise the standard quality and acceptance of other diets and are consumed both as raw/ processed products.Tomato is a good source of vitamin A, Cand are usually consumed as processed foods. Tomatoes are generally considered to be acidic, but their pH depends on their degree of ripeness and variety. In home scale preservation of tomato purees, use of salt and oil as a natural preservative, extends its shelf life for about 2 – 3 days. Poor sanitation and unhygienic practices of processing of tomatoes causes the contamination of diarrheal pathogens. Therefore,in industrial processing of tomatoes, due to its large scale processing, the chemical preservatives such as sodium benzoate and potassium metabisulfite are added into the food mainly to inhibit the bacterial growth and to extend the shelf life of foods (Bourdhrioua *et al*., 2008).

Sodium benzoate is the sodium salt of benzoic acid, which is a carcinogenic additive. When ingested as a food additive it causes obstruction of nutrients at the molecular level, thereby depriving oxygen to the cells. Then the body does not have enough oxygen and nutrients to detoxify causing various diseases such as Parkinson’s neuro-degenerative diseases, premature aging and cancer.

Potassium metabisulfite, also known as potassium pyrosulfite is a white crystalline powder with a pungent sulfur odour. Potassium metabisulfate mainly acts as a chemical sterilant and is used as a food additive. It is also known as E224 (Metcalfe *et al.,* 2003). It is restricted in use, as it may cause allergic reactions in sensitive persons. It may also cause skin irritation, eye irritation, and respiratory irritation.

Therefore, in order to avoid the health hazards of using chemical preservatives in tomato purees and to retard bacterial growth, when stored in PET bottles , the bottles are coated with AgNP (*Glycyrrhiza glabra),* which may serve as an antibacterial agent. In order to enhance the shelf life and preservation of tomato puree without affecting the quality of puree using nanocoats from locally available medicinal plants was attempted in the present study and the nanocoated PET bottles stored with tomato purees were tested for a period of ten days against the enteropathogenic diarrheal species.



Shelf Life of Nanocoated PET Bottles with Tomato Puree

Figure 52

Figure 52 and Table XXII shows the Three way ANOVA (Three way - Analysis of variance) for the shelf-life study of PET bottles, experimental (nanocoated) are coated with 30 µl of (*Glycyrrhiza glabra*) AgNP suspension and control (uncoated) stored with tomato puree, for a period of ten days against the three enteropathogenic diarrheal species.

TABLE XXII

SHELF-LIFE STUDY OF PET BOTTLES WITH TOMATO PUREE AGAINST *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS&R)*

Day of Testing	*E.coli (MTCC 40)*			*S.enterica (MTCC 3219)*			*Sh.dysenteriae (PSGIMR)*		
	E1	**E2**	**Cont**	**E1**	**E2**	**Cont**	**E1**	**E2**	**Cont**
0	NIL	NIL	17	NIL	NIL	12	NIL	NIL	39
2	NIL	NIL	26	NIL	NIL	28	NIL	NIL	65
4	NIL	NIL	53	NIL	NIL	74	NIL	NIL	197
6	NIL	NIL	81	NIL	NIL	118	NIL	NIL	243
8	46	50	113	89	93	162	24	27	TNTC
10	128	131	TNTC	96	98	TNTC	38	36	TNTC
S Ed	0.81								
CD	2.26**								

** Significant at (P< 0.01) level; CD – Critical difference; C – Control

SE – Standard Error ; E1, E2 – Experiment; TNTC – Too Numerous to Count

From 0th day to 6th day, the experimental samples (nanocoated) showed no microbial growth. The control (uncoated) PET bottles showed an increase in the number of bacterial count of 81 against *E.coli (MTCC 40).* On 8th day, it is observed that the experimental (nanocoated) PET bottles showed an inhibition of 57.52 per cent which was reduced to 56.83 per cent on 10th day against the *E.coli (MTCC 40).* To thecontrol (uncoated) bottles exhibited no microbial inhibition. The experimental samples (nanocoated) and control (uncoated) PET bottles against *E.coli (MTCC 40)* showed a statistically significant (P<0.01) level of microbial inhibition compared to the control against *E.coli (MTCC 40).*

From 0th day to 6th day, there is no microbial growth in experimental (nanocoated) PET bottles, when compared to the control (uncoated) which showed a drastic increase in microbial counts upto 118 counts against *S.enterica (MTCC 3219).*However on the 8th day, the experimental group (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*). The control (uncoated) bottles showed no microbial inhibition. The difference between the experimental (nanocoated) and control (uncoated) PET bottles against the *S.enterica (MTCC 3219)* was found to be statistically significant (P<0.01).

No microbial growth was observed from 0th day to 6th day in the experimental (nanocoated) PET bottles, when compared to the control (uncoated) PET bottles which showed a rapid increase in microbial growth of *S.dysenteriae (PSGIMS & R).*There was no microbial growth of S.enterica (MTCC 3219) in the experimental samples compared to the control, which showed a drastic inhibition on 8th day showed a maximum inhibition of 87.5 per cent and on 10th day its inhibitory activity increased to 87.66 per cent against the *S.dysenteriae (PSGIMS & R)* respectively. The experimental samples showed and control group showed that the difference between the values significant (P< 0.01) greater inhibition than control samples during all periods of time.

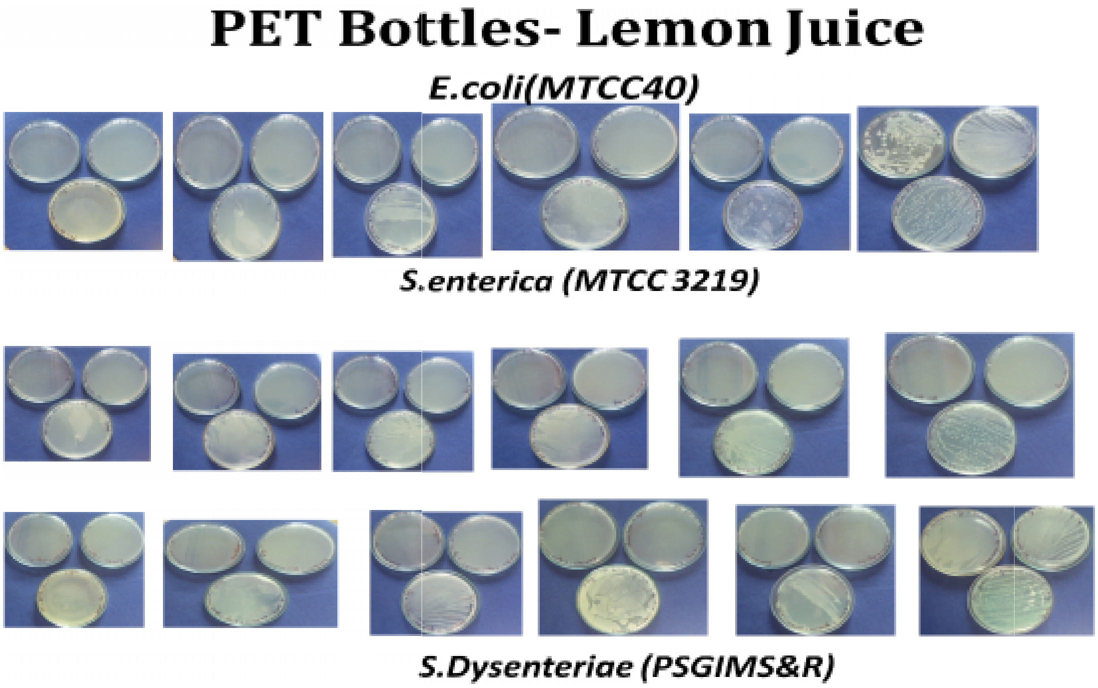
The experimental group of (nanocoated) PET bottles, showed the maximum microbial inhibition against *Sh.dysenteriae (PSGIMS &R),* even after 10 days of storage period, which was significantly higher (P<0.01) than the control (uncoated) samples. There was a statistically significant difference (P< 0.01) between the experimental (nanocoated) and control (uncoated) PET bottles, which might be caused by the microbial inhibition due to the AgNP coating

ii. Shelf Life of Lemon Juice in Nanocoated PET bottles

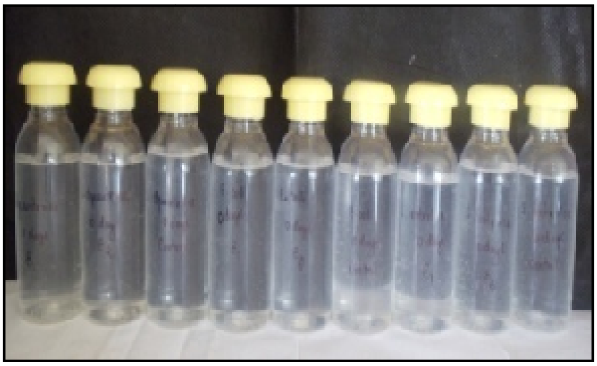
Lemon are non-climateric, with relatively low respiration do not undergo any major softening or compositional changes after harvest and therefore, can normally be stored for relatively long periods of 6–8 weeks (Kader, 2002). The high antioxidant activity of lemon juice is mainly due to ascorbic acid, polyphenols and carotenoids of these compounds (Marlett and Vollendorf, 1994). The ascorbic acid content of lemon juice has been reported to be 680 mg and is mainly affected by its storage period (Sinclair, 1984). Under aerobic conditions long term storage of lemon juices produces the fermentative metabolites (acetaldehyde, ethanol and ethyl acetate) which affect the sensory attributes of lemon juice. The main problem is that lemon cells are ruptured by cutting during processing, which induces the biochemical reactions that shorten the shelf life of lemon juice (Cantwell and Suslow, 2002). The shelf life of lemon juices stored in bottles is relatively short on storage at room temperature. Limiting the growth of undesirable bacteria in lemon juice is one of the main goals in food preservation. The shelf-life of lemon juice is extended by using chemical preservatives namely sodium benzoate for retarding the bacterial growth.

The ill effects of sodium benzoate is that it is bacteriostatic and fungistatic under acidic conditions. It is most widely used in acidic foods such as carbonated drinks (carbonic acid), fruit juices (citric acid) and pickles(vinegar). Tested by the FDA, most of the lemon juice contain benzoate had benzene levels, below 5 ppb and are considered to be dangerous for human consumption by the World Health Organization (2010). The concentration as a preservative is limited by the FDA in the U.S. to 0.1 per cent by weight. In combination with ascorbic acid (vitamin C,E300), sodium benzoate and potassium benzoate form benzene, a known carcinogen. United Kingdom’s Food Standards Agency (FSA) suggests that foods ingested with the sodium benzoate (E211) cause hyperactive behavior (Saltmarsh *et al.,* 2015).

To avoid these sensory changes and health hazards of sodium benzoate as a food preservative in lemon juice, an alternate AgNP (*Glycyrrhiza glabra)* was coated onto the PET bottles mainly to enhance the shelf life of lemon juice without affecting the sensory attributes. Moreover, spoilage of lemon juices occursfrequently by contamination of food borne pathogens such as *E.coli, S.enterica and Sh.dysenteriae.* In order to achieve improved food safety against the food borne pathogens, the shelf life of lemon juices in experimental group (nanocoated) and control (uncoated) PET bottles were evaluated for every two days for a period of ten days stored at room temperature.



Total Viable Counts of PET Material with *Glycyrrhiza Glabra* AgNP against Enteropathogenic Species



Shelf Life of Nanocoated PET Bottles with Lemon Juice

Figure 53

The Shelf Life of PET bottles with lemon juice of experimental (nanocoated) with *Glycyrrhiza glabra* AgNP,and control (uncoated) PET bottles before and after nanocoating were tested for a storage period of ten days against the diarrheal pathogens and are depicted in Table XXIII and Figure 53.

TABLE XXIII

MICROBIAL GROWTH IN *E.coli (MTCC 40), S.enterica (MTCC 3219) AND Sh.dysenteriae (PSGIMS&R)*OFPET BOTTLES WITH LEMON JUICE

Day of Testing	*E.coli (MTCC 40)*			*S.enterica (MTCC 3219)*			*S.dysenteriae (PSGIMR)*		
	E1	**E2**	**Control**	**E1**	**E2**	**Control**	**E1**	**E2**	**Control**
0	NIL	NIL	11	NIL	NIL	7	NIL	NIL	43
2	NIL	NIL	29	NIL	NIL	21	NIL	NIL	89
4	NIL	NIL	63	NIL	NIL	84	NIL	NIL	231
6	NIL	NIL	148	NIL	NIL	189	NIL	NIL	289
8	NIL	NIL	293	NIL	NIL	298	24	27	TNTC
10	67	67	TNTC	NIL	NIL	TNTC	38	36	TNTC
SE	0.66								
CD	1.86**								

** Significant at (P< 0.01) level ; SE – Standard Error; CD – Critical difference; Exp – Experiment; Cont – Control; TNTC – Too Numerous to Count

Table XXIII depicts the shelf life of PET bottles stored with the lemon juice with all the three bacteria inoculated. In experimental group (nanocoated), i.e, PET bottles coated with AgNP, showed no bacterial growth from 0th to 8th day. The uncoated bottles (control group) had TNTC (Too Numerous to Count) with maximum microbial growth for *E.coli (MTCC 40)* and *S.dysenteriae (PSIMS & R)* respectively. On 10th day, the experimental group (nanocoated) PET bottles showed 77.67 per cent of microbial inhibition against the *E.coli (MTCC 40).* The difference between the experimental (nanocoated) and control (uncoated) were statistically analysed and was found to be significant (P <0.01).

Nanocoated PET bottles showed statistically significant (P< 0.01) inhibition against*S.enterica (MTCC 3219),* compared to the control (uncoated) PET bottles (TNTC) which showed >300 microbial counts.

In case of *S.dysenteriae (PSGIMS & R)*, the experimental (nanocoated) PET bottles showed no microbial growth from 0th day to 6th day, when compared to the control (uncoated) PET bottles which showed higher microbial growth.On 8th day, it was observed that the experimental group (nanocoated) had a 91.5 per cent of maximum inhibition and on 10th day, its inhibition decreased to 87.67 per cent against *Sh.dysenteriae (PSGIMS &R)*. During this period, the control (uncoated) had TNTC(Too numerous to count) of microbial counts 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 $P<0.01$.

Among all the three enteropathogenic species, it was observed that the experimental group (nanocoated) PET bottles stored with lemon juice is highly effective against *S.enterica (MTCC 3219)* with no microbial growth till ten days of storage period. On 10th day, the microbial inhibition was 77.67 and 68 per cent against *E.coli (MTCC 3219)* and *S.dysenteriae (PSGIMS&R*) respectively, but there was no *S.enterica (MTCC 3219)* growth till 10 days of storage at room temperature (Figure 54). When these values were statistically analyzed and the difference were found to be significant at ($P<0.01$) level. Hence, nanocaoted PET bottles stored with the lemon juice, which would increase the shelf-life of food products by reducing the bacterial growth significantly.

b. Infant feeding bottles (PP)

The following specification of infant feeding bottles (PP) are used for the present study

Infant feeding bottles (PP) 150 ml

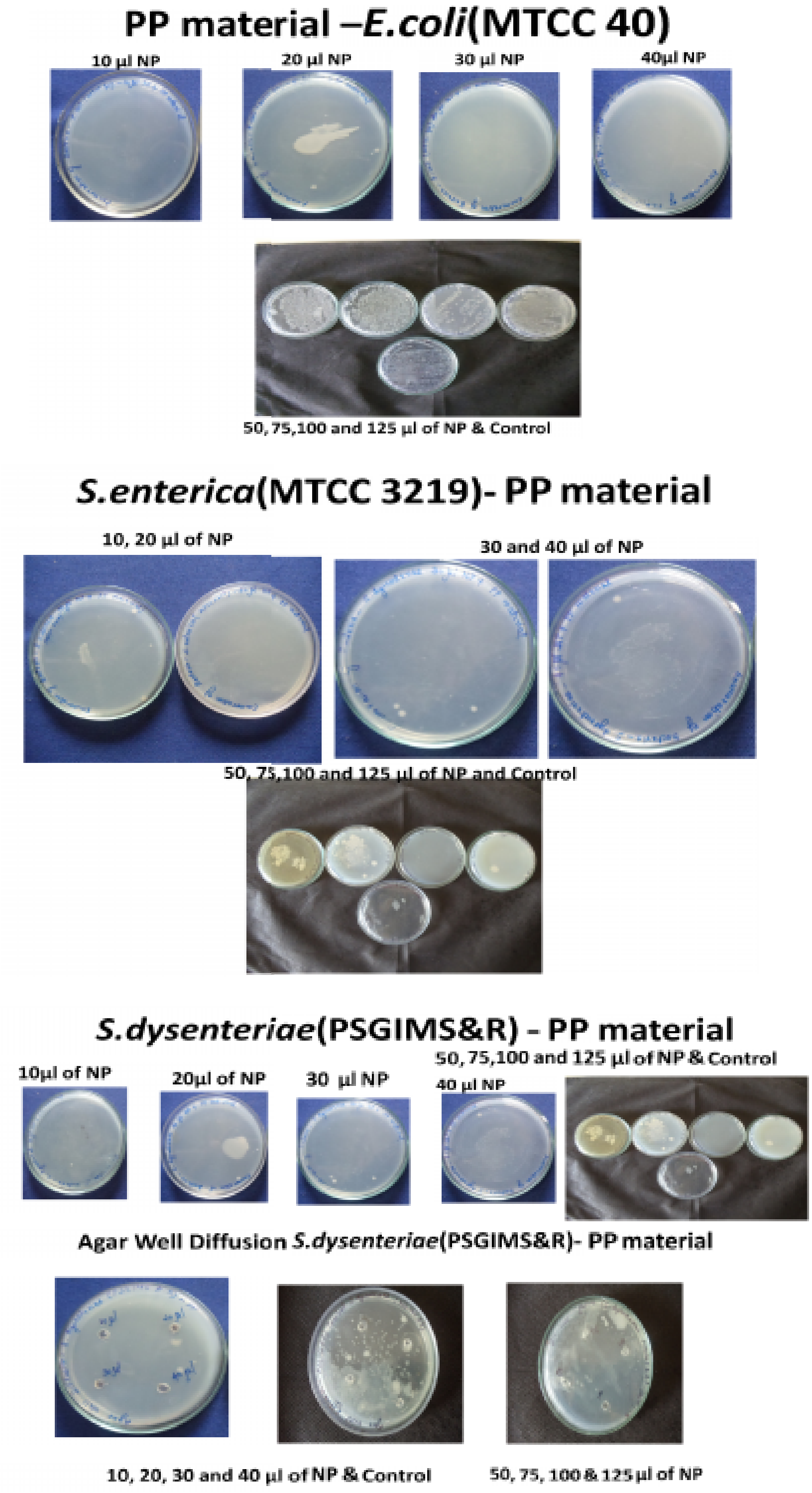
Material – Poly Propylene (PP) food grade
Colour – Multi colour printing
Accessories - hood cap set and latex nipple

Infant feeding bottles have become a popular alternative to glass bottles due to their durability, high resistance and low cost. However, many research studies states that the migration rate of Bisphenol A (BPA) from polycarbonate feeding bottles causes endocrine hormone related problems (Bertsch *et al.,*1999). Therefore, to prevent health risk of infants, other plastic material used in infant feeding bottles, namely polypropylene (PP), an inert material are well suited material for use as freezer to microwave containers (USFDA, 2011). At present, infant feeding bottles made from Poly Propylene (PP) plastic follow the Specification of Indian Food Safety and Standards (Packaging and labelling) Regulations (2011). FSSR reported that the PP feeding bottles stored with foods which follows the IS : 10910 (Indian standards with food safety).

Improper cleaning of infant feeding bottle causes the milk or juice to remain inside the feeding bottles. Milk or juice filled in these contaminated feeding bottles, when fed to the infant cause diarrhea. The replication of bacteria occurs in a two-log phase of bacterial growth cycle in feeding bottles, which accounts for 25 –33 per cent of all deaths among under fives. Due to improper cleansing procedures of feeding bottles, hydrophobic compounds with microbes, still remain in bottles, even after washing, migrating to the food products contained in the feeding bottle. Poor awareness of the mothers, (not following the standard cleansing procedures) causes bacterial residues to remain in bottle and are therefore more critical for infant health (Brody *et al.,* 2008).

Hence in the present study, the infant feeding material (PP) with *Glycyrrhiza glabra*AgNP (experimental) and only PP (control) were tested at varied concentration from 10 to 40 µl and 50 to 125 µl for their antimicrobial activity against diarrheal species *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R) and* are depicted in Figure 54.

Spread plate method is one of the best method for quantifying the microbes on a solid medium. In this experiment, one set of (duplicates) petrid plate – experimental (*Glycyrrhiza glabra* AgNPs from 10 to 40 µl along with PP)and an other set (duplicates) of petrid plates (control) (only PP) were prepared. *Glycyrrhiza glabra* AgNPs of varied concentration from 50 to 125 µl along with PP and a control (only PP). These two controls are used to prevent contamination of microbial cultures and medium in the environment, to maintain the uniform agar growth media and swabbing of microbes for each of the agar petrid plates. These plates are then incubated at 37°C for 24 hrs and the number of colonies are counted. These results are tabulated in Table XXIV



PP material –*E.coli*(MTCC 40)

S.enterica(MTCC 3219)- PP material

S.dysenteriae(PSGIMS&R) - PP material

Agar Well Diffusion *S.dysenteriae*(PSGIMS&R)- PP material

Total Viable Counts of Infant Feeding bottles (PP) Material with *Glycyrrhiza glabra* AgNP against Enteropathogenic Species

Figure 54

TABLE XXIV

ANTIMICROBIAL ACTIVITY OF *GLYCYRRHIZA GLABRA* NANOPARTICLE SOLUTION WITH PP MATERIAL (INFANT FEEDING BOTTLE) *AGAINST E.coli (MTCC 40), S.enterica (MTCC 3219) AND Sh.dysenteriae (PSGIMS&R)*

Concentration (µl)	*E.coli(MTCC 40)*		*S.enterica (MTCC 3219)*		*S.dysenteriae (PSGIMSR)*	
	Count	Percentage	*Count*	Percentage	*Count*	Percentage
10	37	51.32	34	65.66	60	23.08
20	31	59.21	28	71.71	47	39.74
30	22	71.05	20	79.79	34	56.41
40	42	44.74	43	56.56	43	44.87
Control	76	NIL	99	NIL	78	NIL
50	91	14.95	89	17.98	62	27.42
75	78	27.1	82	21.9	60	24.05
100	65	39.25	73	30.48	54	31.64
125	63	41.12	67	36.19	50	36.71
Control	107	NIL	105	NIL	79	NIL
SE	4.02		4.01		3.56	
CD ($p<0.01$)	11.43**		11.41**		10.13**	

** Significant at ($P< 0.01$) level; SE – Standard Error; CD – Critical difference;
Exp – Experiment; Cont – Control

Table XXIV and Figure 54 shows the antimicrobial activity of varied concentration of *Glycyrrhiza glabra* Ag nanoparticles with infant feeding bottles (Polypropylene - PP) materials of both the experimental (coated) and control (uncoated) against enteropathogenic species.

It is observed that 30 µl of Ag NP *(Glycyrrhiza glabra)* showed 71.05 per cent of maximum inhibitory activity against *E.coli (MTCC 40),* when compared to the control (uncoated) PP bottles which showed nil inhibitory activity against the *E.coli (MTCC 40).* The difference between the experiment (nanocoated) and control (uncoated) PP feeding bottles, before and after coating with the AgNPs (*Glycyrrhiza glabra)*was statistically significant at $P< 0.01$.

The statistical analysis between the experimental (coated) and control (uncoated) PP bottles for the antimicrobial activity of *Glycyrrhiza glabra* Ag NP with the PP material against the *S.enterica (MTCC 3219)* showed thatamong the varied concentration of AgNPs, 30 µl showed the 79.79 per cent of highest inhibition against the *S.enterica (MTCC 3219).* Experimental (coated) showed statistically significant inhibitory activity (P< 0.01) over control.

The experimental group (nanocoated) PP bottles with *Glycyrrhiza glabra AgNPs* showed 56.41 per cent (maximum) microbial inhibition against *S.dysenteriae (PSGIMS & R),* when compared to the control (uncoated) PP bottles that had nil microbial inhibitory activity against *S.dysenteriae (PSGIMS & R).* The statistical analysis was done between the experimental (nanocoated) and control(uncoated) PP feeding bottles and the difference was found to be significant at one percent level of significance (P< 0.01).

i. Shelf Life of Milk and Lemon Juice in Nanocoated Infant Feeding bottles (PP)

Infant feeding bottles (PP) serve as an alternative plastic material for feeding infants with liquid foods, due to its containment and convenience. Among liquid foods, milk and diluted fruit juices (lemon juices) are the most commonly used foods for feeding infants using feeding bottles (PP). The optimal environment for bacterial growth on any food item is warmth and moisture. Lemon juice left out at room temperatures between 40 and 140°F ("danger zone" for bacterial growth) is sure to be contaminated. Though, the citric acid in lemon juice acts as a natural preservative and will prevent bacteria from growing quickly, yet it also starts bacterial growth and grow rapidly, when kept for long hours at room temperature. The contaminated lemon juice could possibly lead to food borne illness such as diarrhea. The frequent indicators of food borne illness among infants fed with contaminated foods are diarrhea and abdominal pain.

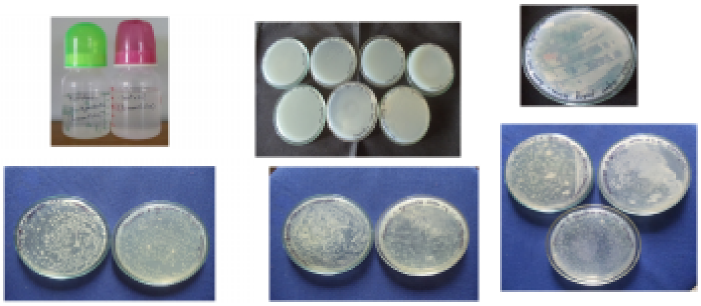
Usually, sodium bisulfite (Sodium hydrogen Sulfite) is used as a chemical preservative to increase the shelf life of lemon juice. It is a white, odorless inorganic salt, used as an additive to enhance the shelf life of foods. It is a chemical additive that preserves the aroma and flavor of bottled lemon juice.

However, the FDA prohibits the use of sodium bisulfite in meats, raw fruits and vegetables which are rich in vitamin B 1 food sources. Sodium bisulfite releases sulfur dioxide gas caused by chemical reactions that are induced by bacteria.

The ill effects of sodium bisulfate causesvarying degrees of dermatological, pulmonary, gastrointestinal and cardiovascular symptoms, including nausea, abdominal cramping, diarrhea, difficult breathing, swelling, itching and reddening of the skin. Allergic reactions are also produced within 15 to 30 minutes after the ingestion of sodium bisulfite. Hence, as seen from the present study, *Glycyrrhiza glabra* (AgNP) could be used as a nanocoating agent in the infant feeding bottles (PP).

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

Infant feeding bottles-Lemonjuice
***S.dysenteriae*- Total Viable Count**



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

Figure 55

Though infant feeding PP bottles are heat resistant, non toxic, compatible and recyclable. Yet, the spoilage of milk in infant feeding (PP) bottle be may due to the transmission of oxygen, light-induced oxidation, enzyme activity and psychrotrophic bacteria, which causes the curdling of milk at higher pH than required for acid curdling. The higher protein, lipid composition and pH neutrality makes the food a highly perishable product with maximum replication of microbial potential, leading to spoilage and reducing shelf life. In addition, the microbes also rapidly multiply on long hours of storage in feeding bottles, thereby causing deterioration of milk. Experimental (nanocoated) PP feeding bottles coated with *Glycyrrhiza glabra* (AgNP) could play a vital role in the storage of milk and afford effective protection against the food borne diarrheal illness. Hence, in the present study, the milk was stored was evaluated in nanocoated PP feeding bottles and tested for its shelf life for every ½ an hour for a period of 3 hours.

TABLE XXV

SHELF-LIFE STUDY OF INFANT FEEDING BOTTLES WITH MILK &LEMON JUICE AGAINST *SH.DYSENTERIAE (PSGIMS&R)*

Time (Hours)	***Sh.dysenteriae (PSGIMS& R)***					
	Milk			**Lemon juice**		
	Exp	**Control**	**Percentage**	**Exp**	**Control**	**Percentage**
0	Nil	169	Nil	Nil	87	Nil
½	Nil	253	Nil	Nil	140	Nil
1	Nil	TNTC	Nil	Nil	224	Nil
1 ½	Nil	TNTC	Nil	Nil	291	Nil
2	23	TNTC	92.33	Nil	TNTC	Nil
2 ½	41	TNTC	86.33	Nil	TNTC	Nil
3	48	TNTC	84	Nil	TNTC	Nil

Milk (alkali food) is filled in infant feeding bottles (PP) of experimental (nanocoated) coated with 30 µl of silver nanoparticles *(Glycyrrhiza glabra)* and control (uncoated) stored separately to test its shelf –life for a period of ten days (Table XXV and Figure 55). The response of microbial growth of *S.dysenteriae (PSGIMS & R)*was tested for every ½ an hour for a period of 3 hours. It was observed that the PP feeding bottles of experimental group (nanocoated) with *Glycyrrhiza glabra* AgNP showed that no microbial growth during till 0 – 1 ½ hour, when compared to the control (uncoated) feeding bottles which had 253 number of microbial counts of the *S.dysenteriae (PSGIMS & R)*whichincreased to TNTC (Too numerous to Count) at 1 hour of storage of milk. The experimental bottles (nanocoated with *Glycyrrhiza glabra AgNP* showed 92.33 per cent of microbial inhibition against the *S.dysenteriae (PSGIMS& R)*during 2nd hours of storage of milk. Its inhibitory activity slightly decreased to 86.33 per cent and 84 per cent at 2½ and 3 hours of milk storage. In the control (uncoated PP feeding bottles, there was no inhibition whatsoever against *S.dysenteriae(PSGIMS&R)* throughout the test period of three hours.The experimental PP feeding bottles (nanocoated) stored with the milk had the maximum microbial inhibition against diarrheal species till 1 ½ hours of storage.

Lemon juice (acidic food) was stored for three hours in nanocoated infant feeding bottles (PP) (coated with 30 µl of AgNP- *Glycyrrhiza glabra)*and uncoated bottles. The microbial activity of *S. dysenteriae (PSGIMS &R)*was tested for every period of ½ an hour. From 0 to 3 hours, no microbial growth was observed in experimental group (nanocoated) PP feeding bottles. The control (uncoated) showed 87 microbial counts in the 1st hour of *S.dysenteriae (PSGIMS&R).* On 2 hrs of storage,microbial counts in uncoated bottles drastically increased to 291 microbial counts of *S.dysenteriae (PSGIMS&R).* Beyond two hours, thefeeding bottles showed (TNTC > 300 microbial counts) of *S.dysenteriae (PSGIMS & R)* till 3 hours of storage period.

It was observed that the milk stored in experimental group (nanocoated) feeding bottles showed the maximum inhibiton of *S.dysenteriae (PSGIMS & R)* at room temperature for a period of 3 hours. There was no microbial growth of *S.dysenteriae (PSGIMS & R)*in nanocoated feeding(PP) bottles stored with the lemon juice for a period of 3 hours.

In experimental (nanocoated) PP infant feeding bottles stored with lemon juice, no microbial growth from 0 to 3 hours, whereas control (uncoated) in infant feeding bottles showed microbes TNTC. Similar findings has been reported in experimental (nanocoated) feeding bottles stored with milk from 0 to 1 ½ hour against *S.dysenteriae (PSGIMS & R),* when compared to that of the control (uncoated). Higher microbial inhibition was observed at 3 hrs of milk stored in experimental (nanocoated) feeding bottles and its inhibiton was 84 percentage (Figure 55) respectively.

Therefore, the inorganic silver nanoparticle possess the highest stability withstanding the processing conditions, which have attracted the attention of scientists over the last decade.

c. Zip lock covers (PE)

The following specification of zip loc covers (PE – Polyethylene) are used for the present study

Material – Poly Ethylene
Package contents – 100 g
Size – 7 cm X 11 cm; 8” X 4.3” (W XL)
Thickness – 2 Mil
Net weight – 50 g
Colour – clear, transparent

Polyethylene is one of the most important packaging materials for storing foods. Zip loc covers(PE) are made up of Low-density polyethylene (LDPE) are manufactured from ethylene monomer using high pressures ranging from 100 to 135 MPa, at temperatures in the range of 150°C to 300°C, in the presence of a small amount of oxygen or an organic peroxide. Zip loc (PE) covers are widely used in laminations, where they provide the inner layer requiring good heat seal ability. In addition, these covers are strong but flexible, tough, chemically inert, have high clarity and are inexpensive. Generally, polyethylenes are characterized by having a low permeability to water vapour, high permeability to oxygen, carbon dioxide and other gases. They are good heat sealers forming a strong seal almost instantly, barrier bags which are ideal for storage of foods sensitive to moisture and oxygen, heat resistant, resealable, compatible with vacuum sealers and are cost effective. Hence, in the present study, shelf life study of tomato puree in nanocoated ziploc covers were evaluated against the diarrheal species namely *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R)* respectively.

Table XXVI and Figure 56 shows the antimicrobial activity of varied concentration of *Glycyrrhiza glabra* nanoparticle with zip lock (PE) materials, before and after coating with AgNP against the namely *E.coli (MTCC 40), S.enterica (MTCC 3219)* and *Sh.dysenteriae (PSGIMS & R).*

TABLE XXVI

ANTIMICROBIAL STUDY OF *Glycyrrhiza glabra* NANOPARTICLE SOLUTION WITH ZIP LOCK MATERIALS *AGAINST E.COLI (MTCC 40), S.ENTERICA (MTCC 3219) AND SH.DYSENTERIAE (PSGIMS&R)*

Concentration (µl)	***E.coli* *(MTCC 40)***		***S.enterica* *(MTCC 3219)***		***S.dysenteriae* *(PSGIMS & R)***	
	Count	**Percentage**	**Count**	**Percentage**	**Count**	**Percentage**
10	51	33.77	48	39.24	37	47.89
20	48	37.66	36	54.43	43	39.44
30	35	54.55	21	73.42	18	74.65
40	57	25.97	51	35.44	49	30.99
Control	77	Nil	79	Nil	71	Nil
50	66	27.47	59	31.4	50	27.54
75	72	20.88	61	29.07	53	23.19
100	74	18.68	65	24.42	56	18.84
125	78	14.29	68	20.93	59	14.49
Control	91	Nil	86	Nil	69	Nil
SE	4.34		4.96		10.73	
CD	12.34**		14.11**		30.54**	

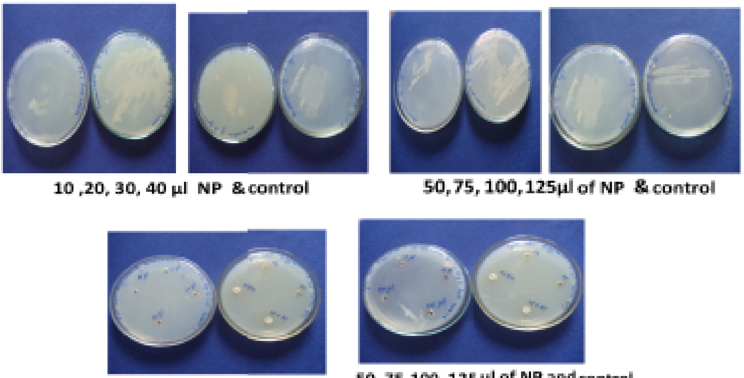
** Significant at ($P< 0.01$) level; SE – Standard Error; CD – Critical difference; Exp – Experiment; C – Control; TNTC – Too Numerous To Count

The Zip Loc materials were cut into well size and were tested against enteropathogenic species for enumeration of bacterial counts. By spread plate method, one set of petrid plate (duplicates) *Glycyrrhiza glabra* AgNPs from 10 to 40 µl along with zip loc (PE) material and control (only PE material) were prepared. The other set of petrid plates (duplicates) from 50 to 125 µl of *Glycyrrhiza glabra* AgNPs along with zip loc (PE) material and control (only PE) were also prepared for the study. These two controls were used mainly to avoid the contamination of microbial cultures, medium in the environment, to maintain the uniform agar growth media and swabbing of microbes for each of the agar petrid plates. The control depended on the choice of the growth medium and test microbes, which are being cultured. The experimental (nanocoated) PE material with known concentration of *Glycyrrhiza glabra* AgNP (from 10 to 40 µl, 50 to 125 µl) and along with PE material and control (uncoated) are swabbed and spread with microbial culture over the surface of the agar plate, using a sterile glass spreader. The control mainly depends on the choice of the growth medium and the test microbes, which are being cultured. The petrid plates were then incubated at 37 °C for 24 hours and then the zone of inhibition was recorded. It was found that 30 µl of *Glycyrrhiza glabra* AgNP had the best antimicrobial activity and 50 µl had the poor antimicrobial activity (Figure 56).

In the case of zip loc covers (PE) material of experimental group (nanocoated), among which 30 µl of AgNP showed 54.55 per cent of maximum inhibitory activity against the *E.coli (MTCC 40),* when compared to the control (uncoated) zip loc covers (PE) which showed no microbial inhibition against the *E.coli (MTCC 40)* (Figure 56). Therefore, zip loc covers after coating with the *Glycyrrhiza glabra (AgNP)* were found to be significantly superior ($P< 0.01$) as microbial inhibitors.

Statistical analysis of the antimicrobial activity of *Glycyrrhiza glabra AgNP* with PE material against the *S.enterica (MTCC 3219)* (Figure 56). Nanocoated zip loc covers of experimental group showed that among the varied concentrations of AgNP, 30 µl had the highest (73.42 per cent) inhibition against the *S.enterica (MTCC 3219)*, when compared to the control (uncoated) which showed least microbial inhibition against the *S.enterica (MTCC 3219)*. The difference between the experiment (nanocoated) and control (uncoated) zip loc covers (PE) is found to be statistically significant at ($P < 0.01$).

Zip & lock - *E.coli(MTCC 40)*



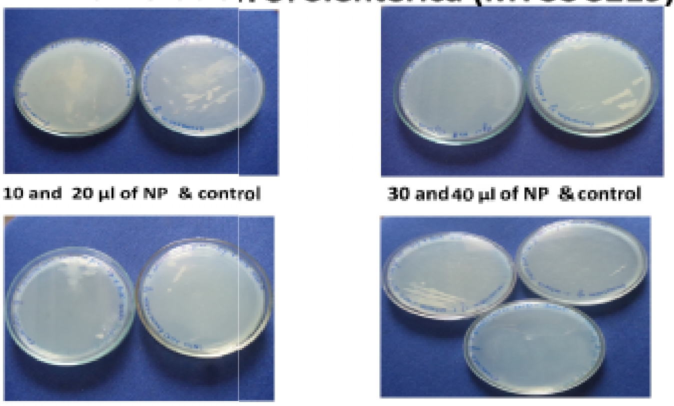
10 ,20, 30, 40 µl NP & control

50, 75, 100, 125µl of NP & control

10, 20 30 , 40 µlNP and control

50, 75, 100, 125 µl of NP and control

Enumeration of S.enterica (MTCC 3219)



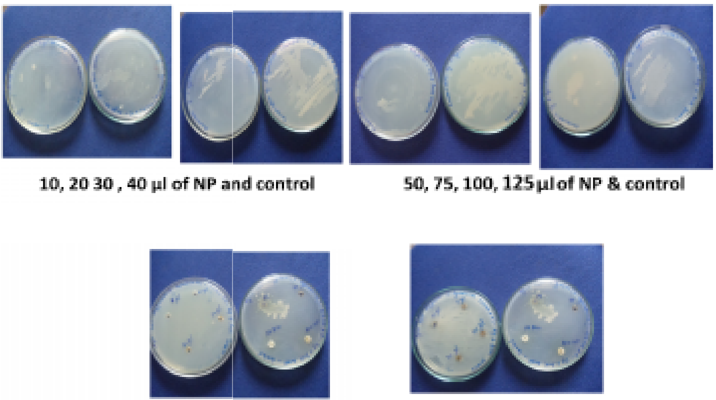
10 and 20 µl of NP & control

30 and 40 µl of NP & control

50 and 75 µl of NP & control

100, 125 NP & control

Zip & Lock - *S.dysenteriae* (PSGIMS&R)



10, 20 30 , 40 µl of NP and control

50, 75, 100, 125µl of NP & control

10, 20 30 , 40 µl of NP and control

50, 75, 100, 125µl of NP & control

Total Viable Counts of Zip Loc material with *Glycyrrhiza glabra* AgNP against Enteropathogenic species

Figure 56

Of the varied concentration of *Glycyrrhiza glabra* silver nanoparticles from 10 to 125 µl AgNP, along with the zip loc covers (PE), among which 30 µl showed the maximum microbial inhibition of 74.65 per cent of inhibition against *S.dysenteriae (PSGIMS &R)* respectively(Figure 56) covers coated with 30 µl of silver nanoparticles *(Glycyrrhiza glabra)* had the best antimicrobial activity and 125 µl had the poor antimicrobial activity, when tested against the enteropathogenic species. Statistical analysis revealed that difference between coated and uncoated covers was statistically significant ($P< 0.01$).

i.Shelf Life of Tomato Puree in Nanocoated Zip Loc Covers

Shelf life of tomato puree (zip loc covers) before and after nanocoating were tested against the diarrheal species for a period of ten days and is depicted in Table XXVII and in Figure 57.

TABLE XXVII

SHELF-LIFE STUDY OF ZIP& LOC COVERS WITH TOMATO PUREE AGAINST *E.coli (MTCC 40), S.enterica (MTCC 3219) AND Sh.dysenteriae (PSGIMS&R)*

Days of testing	*E.coli (MTCC 40)*				*S.enterica (MTCC 3219)*				*Sh.dysenteriae (PSGIMS & R)*			
	E1	**E2**	**C**	**%**	**E1**	**E2**	**C**	**%**	**E1**	**E2**	**C**	**%**
0	Nil	Nil	Nil	NIl	Nil	Nil	12	Nil	Nil	Nil	18	Nil
2	Nil	Nil	46	Nil	Nil	Nil	31	Nil	Nil	Nil	73	Nil
4	Nil	Nil	71	Nil	Nil	Nil	57	Nil	Nil	Nil	101	Nil
6	Nil	Nil	183	Nil	Nil	Nil	104	Nil	Nil	Nil	265	Nil
8	46	50	TNTC	84	63	63	238	73.53	24	27	TNTC	91.5
10	128	131	TNTC	56.83	110	110	TNTC	57.57	38	36	TNTC	87.67
S E	0.96											
CD	2.69**											

**Significant at ($P< 0.01$) level; SE– Standard Error; CD – Critical difference

Exp – Experiment; Cont – Control

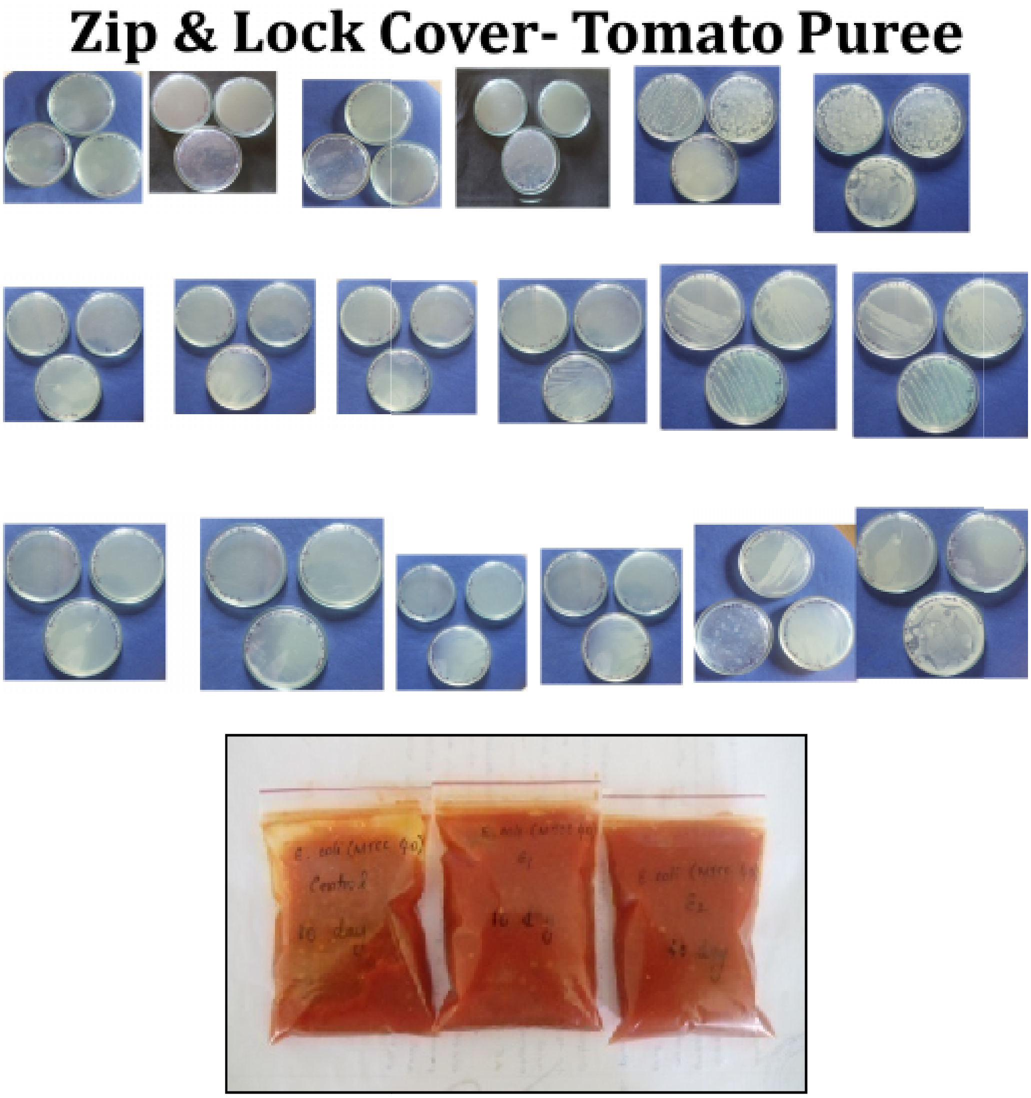
From 0th day to 6th day, the experimental group (nanocoated) zip loc covers (PE) stored with tomato puree showed no microbial growth of *E.coli*

(MTCC 40), when compared to the control (uncoated) zip loc covers (PE) harboured higher microbial growth. On 8th day, zip loc covers of experimental group (nanocoated) had 84 per cent microbial inhibition against the *E.coli (MTCC 40)* and the inhibition delcined to 56.83 per cent. On 10th day, probably due to the higher moisture content of tomato puree the microbial load increased. Nanocoated zip loc covers were superior to the uncoated zip loc covers (PE). The difference between them was significant (P<0.01).

It was also observed that from 0th day to 6th day, that there was no microbial growth in experimental group (nanocoated) zip loc covers, than the control (uncoated) which had higher microbial counts of 104 against the *S.enterica (MTCC 3219)*. On 8th day, the experimental group (nanocoated) zip loc covers had 73.53 per cent of microbial inhibition. On 10th day, its inhibitory activity decreased to 57.57 per cent against the *S.enterica (MTCC 3219)* than the control (uncoated) zip loc covers had TNTC (Too Numerous to Count) on 10th day of storage period of time. Statistical analysis is done between the experimental (nanocoated) and control (uncoated) and the difference is found to be significant at (P <0.01) level.

Tomato puree stored in Zip loc covers of experimental group (nanocoated) with AgNPs showed that from 0th day to 6th day, there was no microbial growth as compared to the control (uncoated) for the *Sh.dysenteriae (PSIMS & R).* Zip loc covers of control group showed an increase of 265 microbial counts against the *Sh.dysenteriae (PSGIMS & R).* On 8th day, the experimental group (nanocoated) zip loc covers showed 91.5 per cent of maximum inhibition and on 10th day, its inhibitory activity declined to 87.67 per cent against the *Sh.dysenteriae (PSGIMS & R)* respectively. The difference between these experimental (nanocoated) and control (uncoated) were statistically analyzed and are found to be significant (P<0.01).

Among the three enteropathogenic species, it was observed that tomato puree stored in experimental group (nanocoated) zip loc covers showed a maximum of 87.67 per cent of microbial inhibition and were found to be highly effective, when compared to the control (uncoated) zip loc covers which showed higher microbial growth (TNTC) against the *Sh.dysenteriae (PSGIMS & R)* during the storage period of ten days. The silver nanoparticles were found to be highly effective against the diarrheal species. Therefore, these nanoparticles coated onto surface of the food packages inhibit the growth of microbial pathogens.



Shelf Life of Nanocoated Zip Loc Covers With Tomato Puree

Figure 57

Zip Loc covers (PE) of polyethylene films coated with silver nanoparticles had a higher antimicrobial activity against the enteropathogenic species of gram negative bacteria. This may be due to the surface modification of polyethylene films with the polar functional groups like C\O and CO are formed by impregnation of AgNPs. This causes an increase in silver ions release and in aqueous media. The presence of these polar groups increases the hydrophilicity and reactivity of polymeric films, against diarrheal pathogens. Therefore, these AgNPs could be used as a nanocoating to enhance the shelf life of foods.

It was also observed that the coating of AgNPs onto the polymer of food packages, rapidly diffuse from the surface of the polymer into the food. The coating of nanoparticles which poses silver ions as a biocide substance are released into the food packages, thereby enhancing the shelf life of foods. The AgNPs act as antimicrobial substances, which would reduce the viability of microbes, thereby reducing the microbial growth, extending the shelf life of foods and its safety. These AgNP *(Glycyrrhiza glabra)* are incorporated at a low concentration and show the highest antimicrobial activity with the current food legislation. According to the European Food Safety Authority, the restricted migration of silver to foodstuffs to 0.05mg/kg food (EFSA) showed a low threshold, which would limit the usage of silver as an antimicrobial agent in the food packaging sectors. Therefore, coating of silver nanoparticles at <1% concentration of Ag onto low-density polyethylene packages would extend the shelf-life of foods than the control (uncoated) PE films.

On the whole, from the present study, it was observed that the experimental group (nanocoated) with *Glycyrrhiza glabra* AgNP and control (uncoated) onto commercially available packages namely PET, infant feeding bottles (PP) and zip loc covers (PE) were analyzed for antibacterial activity. By comparing the antibacterial activity of experimental (nanocoated) and control (uncoated) of all the three food packages. It was observed that the experimental group (nanocoated) of PET bottles stored with the tomato puree showed a maximum inhibition of 87.66 per cent against the *S.dysenteriae (PSGIMS & R)*tilla storage period of ten days. On 0–3 hours of storage of lemon juice in experimental (nanocoated) PP feeding bottles there was no microbial growth, while control (uncoated) feeding bottles had TNTC. Similar findings have been reported in experimental (nanocoated) and control (uncoated) PP feeding bottles stored with milk against *S.dysenteriae (PSGIMS & R).* Higher microbial inhibition of 84 per cent was observed until three hrs of storage period. Therefore, these silver nanoparticles are highly effective as a coating onto the PET, Infant feeding bottles and Zip loc covers to inhibit the growth of diarrheal pathogens.

Antibacterial mechanism of AgNP *(Glycyrrhiza glabra)*

Not much research has been done on the shelf life study of *Glycyrrhiza glabra* AgNPs coated on the commercially available food packages namely PET bottles, infant feeding bottles (PP) and zip loc covers (PE). Hence, in the present study, the nanocoating of AgNPs and its possible antibacterial mechanism against gram negative species are discussed here. The detailed mechanism of Ag nanoparticles as an antimicrobial agent, still remains unclear.

Bourdhrioua *et al.,* (2008) illustrates that the interaction of Ag nanoparticles with oxygen results in enhanced antimicrobial activity, compared to that of the silver metal. In addition, it is observed that the oxidation state of the Ag atom in silver nanoparticles are the basic fundamental property to achieve its antimicrobial property. The antimicrobial activity are mediated only by Ag+ chemisorbed on the surface of Ag nanoparticles. Therefore, zero-valence Ag nanoparticles do not display appreciable biological activity, which are incorporated as a coating onto packaging materials. It was also observed that the enzyme, microbes and water activities are the most important factors and affect the shelf-life of foods. Hence, these silver nanoparticles are used as a coating onto food packages, which would act as barrier against physical and chemical damages, thereby reducing the spoilage of foods in nanocoated packages. It is noted that food stored in these nanocoated containers are found to inhibit the microbial growth and prolong the shelf life of foods up to 10 days, compared to those foods stored in uncoated (conventional containers) which had a higher microbial growth and a lesser shelf life. Therefore, nanocoated packages act as antimicrobial agents in maintaining the quality and food safety during storage.

As discussed earlier from phase I, FRAP antioxidant showed that *Glycyrrhiza glabra*AgNP had the maximum antioxidant potential of 520 µg, when compared to other AgNP synthesised from other medicinal plants.Therefore, the high antioxidant content could be due to rich phyto constituents. The phytochemical screening of *Glycyrrhiza glabra* silver nanoparticles by thin layer chromatography (TLC) showed the presence of phenolic compounds with a purple colour. It is observed that the reactive oxygen species (oxygen/ hydroxyl radicals) are generated by silver nanoparticles (*Glycyrrhiza glabra*) possessing the antioxidants of phenols and thiols which may play a vital role in killing of gram-negative bacteria. The presence of antioxidant prevents the formation of silver oxide on the nanoparticle surface forms the Ag^+ reservoir as a potent antimicrobial agent. The cell wall of gram negative bacteria is disrupted by functional groups. As discussed from phase I, FTIR of the raw powder and AgNP *(Glycyrrhiza glabra)* encapsulated were analyzed for the functional group for its antimicrobial activity.

It is observed that 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 and thiols which are involved in reduction and capping of silver ions leading to effective chelation of silver nanoparticles.Thiols and polyphenols are the two major phytocomponents that induce antioxidant potential. The *Glycyrrhiza glabra* AgNP shows a prominent and a stronger and a narrow peak at 578.06 cm^{-1} corresponding to phenol with maximum antioxidant potential, while the same peak is weak in *Leucas aspera* corresponding to its poor antioxidant potential. Hence the high antioxidant property of *Glycyrrhiza glabra* may be attributed to phenols.A peak observed at 702.65 cm^{-1} and 715.87 cm^{-1} confirms the presence of alky halide contributes for its antimicrobial property.The elemental analysis of *Glycyrrhiza glabra*showed that the silver content is about 0.64 per cent atomic weight. It is as a potent chelating and triggering microbial inhibiting agent, along with the phytochemicals of the plant extract. The highest level of cationic impurity is observed in *Glycyrrhiza glabra* silver nanoparticleswith impurity such as sodium(41.75 per cent). Anionic impurity namely Chlorine and Sulphur of 35.78 per cent and 1.86 per cent respectively are also present. The higher percentage of impurities of Na and Cl of *Glycyrrhiza glabra* are due to the soil rich in minerals, which may vary based on its varied geographical location. The FTIR peak observed at 600 cm^{-}1 and 760.77 cm^{-1}, (raw extracts) shows a shift from 625.29 cm^{-1} to 715.87 cm^{-1}, (AgNP) confirms the presence of alkyl halide, which attributes to the antibacterial action of nanoparticles. This corresponds to the chlorine (Cl) observed in the EDX forming bond with silver nanoparticles.

Cho *et al.,* (2005) state that silver nanoparticles cause intracellular structural and nuclear membranes changein bacterial cell walls leading to denaturation of DNA and RNA, which inhibit replication of bacterial cells. In addition, denaturation of nucleic acid poses the inhibition of cytosolic protein and mitochondrial respiration, leading to bacterial cell death. It is observed that there are three possible antibacterial mechanisms of silver nanoparticles due to their binding onto the bacterial surface, altering the membrane properties. The small size and extremely large surface area of nanoparticles enables them to make strong contact with the microbial surface. The dissolution of silver nanoparticles releases Ag+ ions, which interact with sulphur-containing proteins in the bacterial cell wall, thereby acting as a antimicrobial agent. These silver nanoparticles penetrate deep inside the bacterial cell and lead to DNA damage. The inhibition of microbes are correlated with the silver nanoparticles less than 50 nm, which can be easily transported through cell membrane. Uncharged silver nanoparticles are more toxic than any other form of silver nanoparticles. The interaction of dissolved Ag+ ions with phenol and thiol group, leadto interruption of respiration and proton motive forces of the bacterial cell wall. The disruption of proton motive forces in bacteria cell, inhibits the movements of protons from inside to outside of bacteria. This is needed for ATP synthesis to occur. Hence, if these proton processes are inhibited, the energy-dependent reactions cannot be synthesized which leads to microbial cell death.

The synergistic effect of antibacterial activity of silver nanoparticles are caused by binding and accumulation of silver ions to the cell wall, which causes the inhibition of the bacterial cell. The silver ions interact with phosphorus containing compounds such as DNA of bacterial cell wall. In addition, due to the interference with DNA replication process bacterial proliferation is inhibited over a period of time which mainly depends on the exposure to silver ions. The shape of silver nanoparticles also governs their antibacterial properties.

The antibacterial mechanism of AgNP *(Glycyrrhiza glabra)* may be due to binding and migration into bacterial cells, damaging DNA and its genes. This leads to cell-death. The synergistic bactericidal effects of encapsulated silver nanoparticles, interact and release silver ions to the bacterial cell membrane, leading to cell death of bacteria. The silver ions bind to thiol/ sulfhydryl groups of proteins, leading to inactivation of these proteins. In addition, these intracellular silver nanoparticles cause damage to proteins and nucleic acids inside the bacterial cell. Another mechanism of AgNP (*Glycyrrhiza glabra)* may be due to silver ions that directly bind to the gram negative cell wall of bacteria, leading to accumulation of silver ions inside the cells, thereby causing the leakage of the cellular components, resulting in cell death of gram negative bacteria. Thus, AgNP *(Glycyrrhiza glabra)* serve as a potent antidiarrheal agents, when impregnated as coating onto the commercially available food packaging materials.

Phase III

Synthesis, Standardization and Characterization of Nanogranular Edible Films

A. Synthesis and Standardization of Silver nanoparticles (Glycyrrhiza glabra) edible film

B. Characterization of Edible films

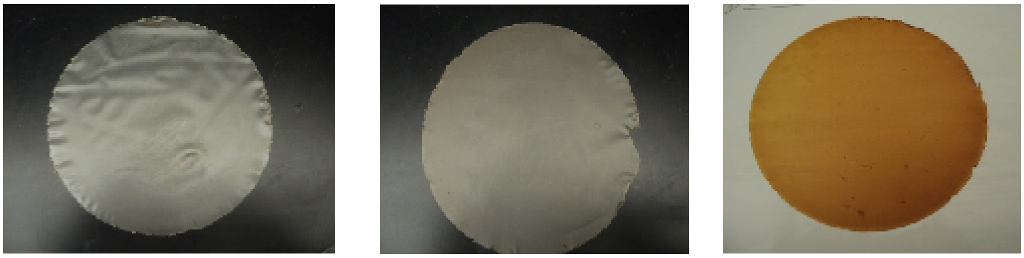
1. Thermal analysis by Differential Scanning Calorimetry (DSC)
2. Thermo Gravimetric Analysis (TGA)
3. X Ray Diffraction (XRD)
4. Water Vapour Permeability (WVP)
5. Antibacterial Potential of Edible Films

1. Synthesis and Standardization of Silver nanoparticles (*Glycyrrhiza glabra)* Edible films

In the last few decades, nanotechology in antimicrobial packaging is emerging as a novel technique with a pivotal role in food safety to prevent food spoilage and extend the shelf-life of foods. Nowadays, most of the wrapped films are made from synthetic polymers. This could lead to extensive problem during degradation and recycling of these polymers. Studies reveal that during nano composite films containing antimicrobial agents possess improved mechanical, thermal, optical and physicochemical properties (Tunc *et al.,* 2011). Antimicrobial packaging is a propitious form of active packaging in which antimicrobial agents agents may be coated, incorporated, immobilized, or surface modified onto packaging materialsto improve safety and shelf-life of food products. Diffusion of the nanoscaled antimicrobial agents on the surface of the food has a crucial role in deploying microbes.

Starch is considered as the most encouraging biodegradable material, as it is inexpensive, easily available and compostable without toxic residues. Hence in the present study, there is an uprising attention in the field of research, to synthesize bio based edible films i.e. starch loaded with silver nanoparticles which show low toxicity and high antioxidant and antimicrobial property. 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 per cent silver with *Glycyrrhiza glabra* nanoparticles respectively (Figure 59) was attempted. Further, these AgNP edible films were tested for their thermal and antibacterial property

Teflon plate



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

Edible Films of 0.01, 0.05 and 0.1 per cent *Glycyrrhiza glabra*AgNP

Figure 58

2. Characterization of Edible Films

i. Film Thickness

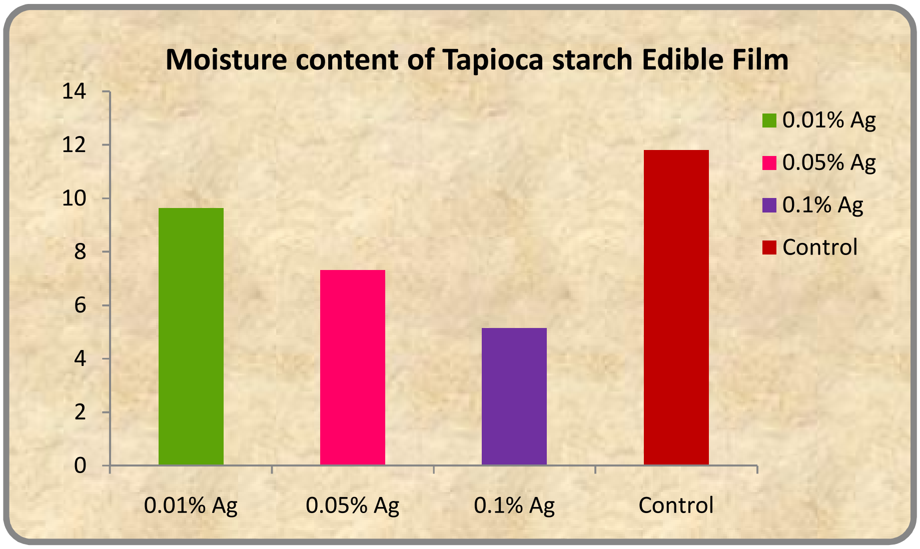
Table XXVIII and Figure 58 present the thickness of the edible film impregnated with nanosilver *(Glycyrrhiza glabra).*

TABLE XXVIII

THICKNESS OF EDIBLE FILM

Silver Impregnated Edible Film (%)	Thickness (µm)	Moisture content (%)
0.01	0.3	9.65
0.05	0.4	7.32
0.1	0.4	5.14
Control	0.3	11.80
SE	0.17	
CD	0.58	
F value	0.22	
F Probability	NS **	

NS ** – Not Significant; SE – Standard Error; CD – Critical difference



Moisture Content of Tapioca Starch Film with 0.01, 0.05 and 0.1 per cent Ag

Figure 59

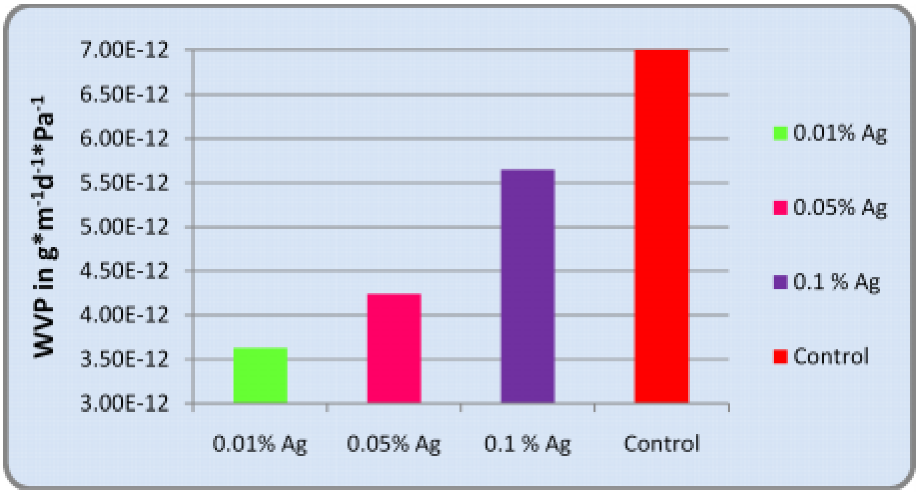
The thickness of the Ag impregnated starch films were calculated from five readings taken from the films. The thickness of the film ranged from 0.3 mm for the 0.01 per cent Ag films to 0.4 mm for 0.05 and 0.1 per cent Ag impregnated films. The thickness did not increase significantly, after the addition of 0.05 and 0.1 per cent Ag respectively.

Table XXVIIIalso depicts the ANOVA of edible film synthesized from *Glycyrrhiza glabra* AgNP and this exhibits the interaction between the silver nanoparticles and starch. However, it was observed that by increasing the AgNP concentration, due to the provision of more dissolved solids in film solution, slightly thicker films were formed. Hence, in the present study, increased film thickness may be due to the interaction of starch with the AgNP in the colloidal suspension. The film thickness is also dependent on both the film composition and processing parameters where the glycerol act as a plasticizer in film processing, which would bind with starch to form starch polymer and the starch-starch bond is replaced by the starch-glycerol-starch bond, resulting in the larger thickness of edible film. The film forming starch solution with higher concentration of silver nanoparticles produces relatively higher dry matter content, leading to a thicker film. Further, due to higher concentration of silver nanoparticles in edible film , there is a greater potential to absorb moisture which causes swelling of the starch granules particles (gelatinization) which produces the film of larger thickness. Glycerol as a plasticizer in processing of edible film, interacts with the film polymer, improving the film thickness. Galus *et al.,* (2013) report that the increase in film thickness is attributed to the weakening of the interchain forces provoked by the interactions of nanoparticles with the starch polymer chains.

The moisture content of the tapioca films with Ag nanoparticles of 0.01, 0.05 and 0.1 per cent Ag were measured and are shown in Table XXVIII and Figure 59. These results revealed that tapioca starch (control) had the highest moisture content of 11.80 per cent and 0.1 per cent Ag film had the lowest moisture content of 5.14 per cent. It was also observed that the moisture content of edible film decreases from 9.65 to 5.14 per cent, with increase in incorporation of Ag nanoparticle from 0.01 per cent Ag to 0.1 per cent Agof the edible film. This may be due to the specific interactions that are linkedto hydroxyl groups in starch and nanoparticles polymer–water interactions by hydrogen bonding, causing a decrease in moisture content of these edible films.

ii. Water Vapor Permeability

The Water Vapor Permeability (WVP) of the Ag impregnated edible films are shown in Figure 60 and Table XXIX.



Water Vapour Permeability of Ag Impregnated Edible Films

Figure 60

The WVP of the tapioca film without nanoparticles (control) showed the highest water vapour transmission of 6.60 X 10^{-11}g×m^{-1}s-^{1}Pa^{-1}. The effect of Ag nanoparticle concentration on WVP of edible films were studied (Figure 60). 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×m-^{1}× s^{-1}× Pa^{-1} respectively, when compared to the WVP of the control (tapioca starch) was 6.60 x 10^{-11} gx$m^{-1}$$s^{-1}$$Pa^{-1}$. There was a statistically significant decrease ($p<0.05$) in the WVP with the addition of Ag nanoparticles, when compared to the control (tapioca starch).

TABLE XXIX

WATER VAPOUR PERMEABILITY OF EDIBLE FILMS

Edible films (% Ag)	WVTR (g/s)	R_H (%)	ΔP (Pa)	WVP (g Xm^{-1}X s^{-1} X Pa^{-1})
0.01 %	1.87 X 10^{-6}	46.1	3.363 X 10^{3}	5.65 X 10^{-12}
0.05	1.40 X 10^{-6}	46.1	3.363 X 10^{3}	4.24 X 10^{-12}
0.1	1.20 X 10^{-6}	46.1	3.363 X 10^{3}	3.63 X 10^{-12}
Control	7.71 X 10^{-6}	46.1	3.363 X 10^{3}	6.60 X 10^{-11}

In the present study, a significant decrease in WVP areobserved with increase in concentrations of Ag nanoparticles. This may be due to the decrease in diffusion coefficient of water in which the Ag nanocrystals were well distributed. That is permeation of water molecules through these tapioca starch films, becomes difficult resulting in a decrease in the WVP values. However, the incorporation of nanoparticles into tapioca starch prevents the formation of hydrogen bonding between starch molecules and water, resulting in a more compact structure with smaller inter-chain spaces which reduce the water vapor diffusion through the film. Further, the reduced WVP of Ag nanoparticles impregnated film may be due to the crystallization of amylose chains in these nano impregnated edible films. AgNP incorporated film decreases the water vapour permeability probably due to the obstruction of intermolecular hydrogen bonds between the polymer. Incorporation of Ag nanoparticles into tapioca starch film causes the interaction of biopolymer network between nanoparticles and starch providing a large number of hydroxyl groups for metal complexation, which may prevent the water penetration to diffuse into the film, thereby improving the barrier property of film.

It was observed that the phenolic functional group of the silver nanoparticle *(Glycyrrhiza glabra)* is hydrophobic in nature and undergoes crystallization. Hence, the tapioca starch film iimpregnated with silver nanoparticles causes the complexity of material with higher density of –OH groups on the surfaces of Ag nanoparticles, in which the crystalline zones of hydrolyzed starch interacts with the silver nanoparticles and glycerol. As a result, more –OH groups of the film network interact with moisture producing a difficult path for water molecules to pass through the film, resulting in a decrease in WVP. Presence of phenolic functional group in 0.01, 0.05 and 0.1% Ag film of *Glycyrrhiza glabra* nanoparticles enhances the crosslinking effect in the film and lead to low moisture and WVPR content. Therefore, the impregnation of higher concentration of Ag nanopartilce with tapioca starch showed a significant decrease in WVP and aquires good barrier property. Hence, Ag impregnated tapioca starch film have a great potential for antimicrobial packaging, which could be used as a film wrapping in food packages to increase the shelf life of foods.

iii. Thermal analysis by Differential Scanning Calorimetry (DSC)

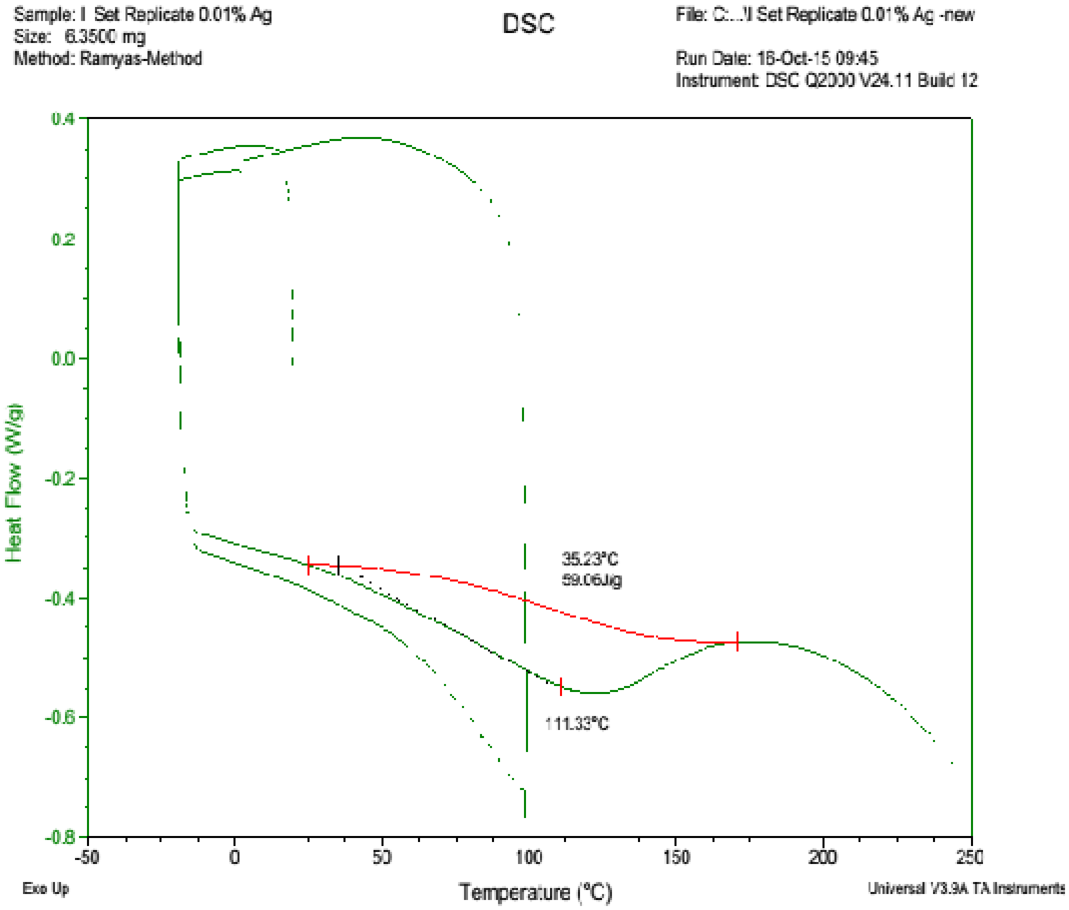
DSC is a thermoanalytical technique in which heat is required to increase the temperature of a sample as a function of temperature. The basic principle is that, when a sample undergoes physical transformation such as phase transition, less or more heat is needed to allow flow to it to maintain both at the same temperature. This is due to the absorption of heat by the sample, as it undergoes the endothermic phase transitions from solid to liquid and the curve denotes the enthalpies of transitions.

Figures 61, 62, 63 and 64 reveal that the thermally induced endothermic transitions are observed for edible films incorporated with silver nanoparticles at three different concentrations of 0.01, 0.05 and 0.1 per cent of silver nanoparticles. The enthalpies of melting and thermal information are shown in Table XXX.

TABLE XXX

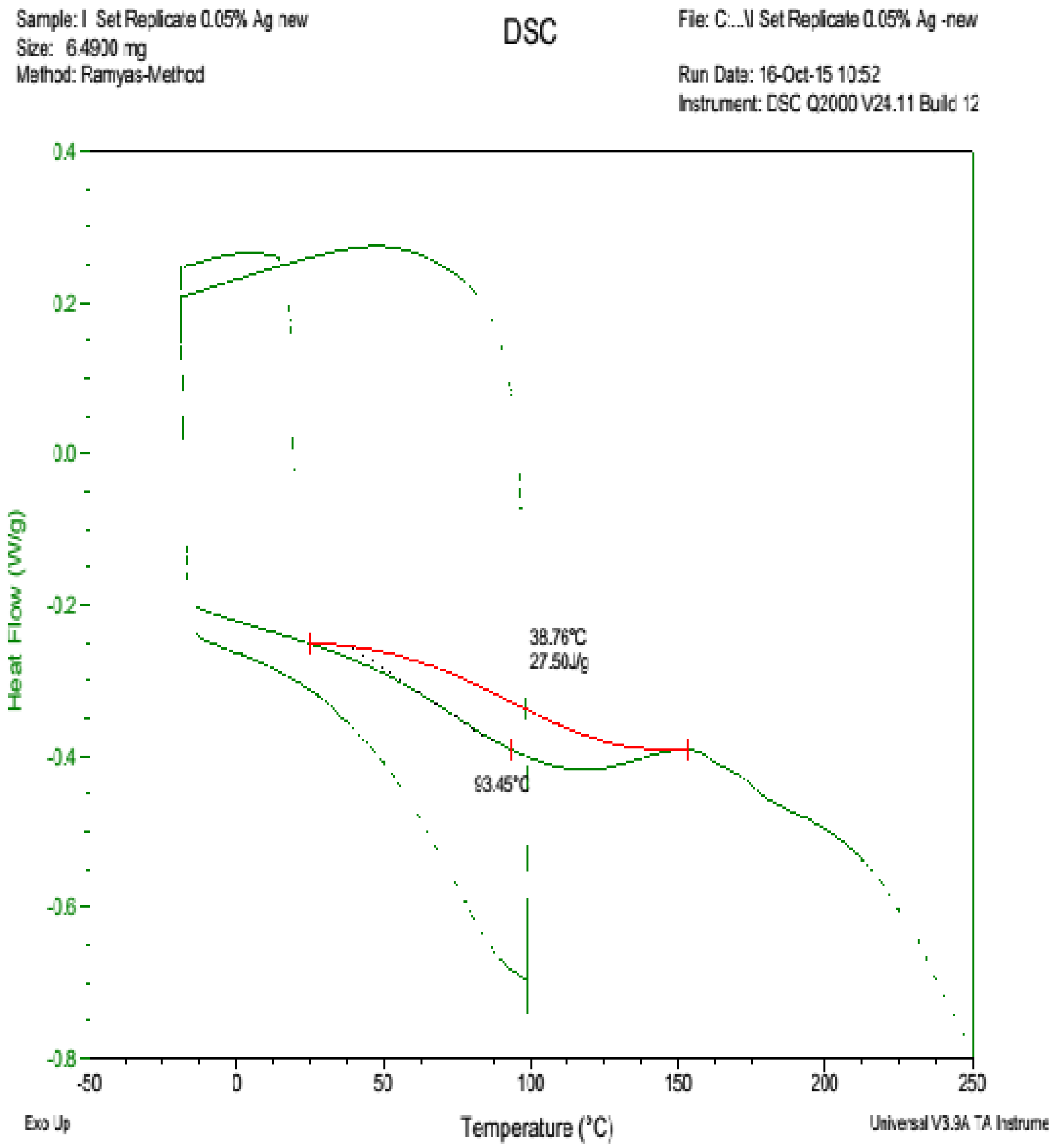
THERMAL DATA FROM DSC

Silver Concentration Edible Films	T_m (°C)	Melting Enthalpy (ΔH) J/g
0.01	111.33	59.06
0.05	93.45	27.50
0.1	117.12	71.88
Control	126.28	113.70



Differential Scanning Calorimetry of 0.01 per cent AgNP *(Glycyrrhiza glabra)*

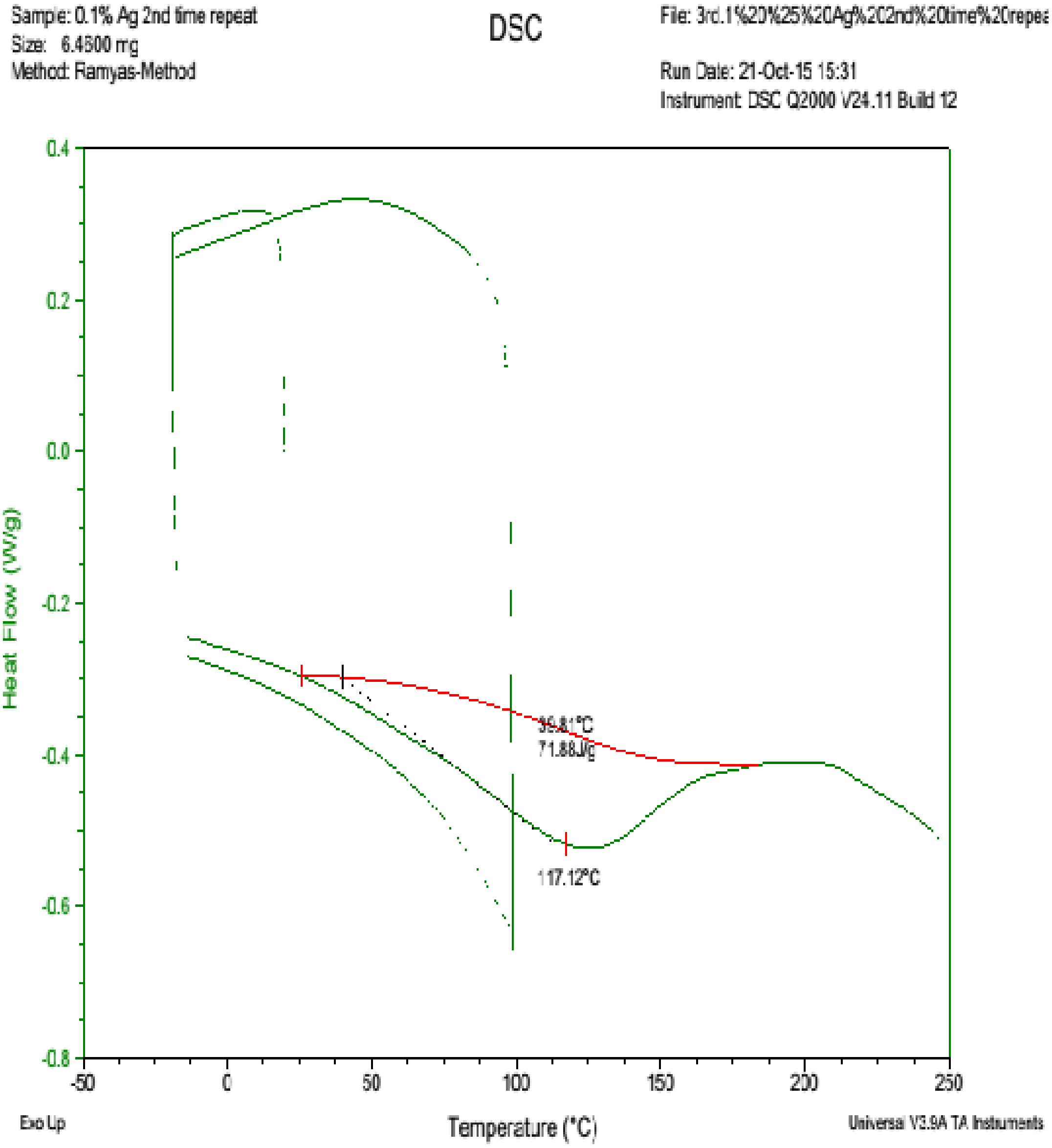
Figure 61



Differential Scanning Calorimetry of 0.05 per cent AgNP

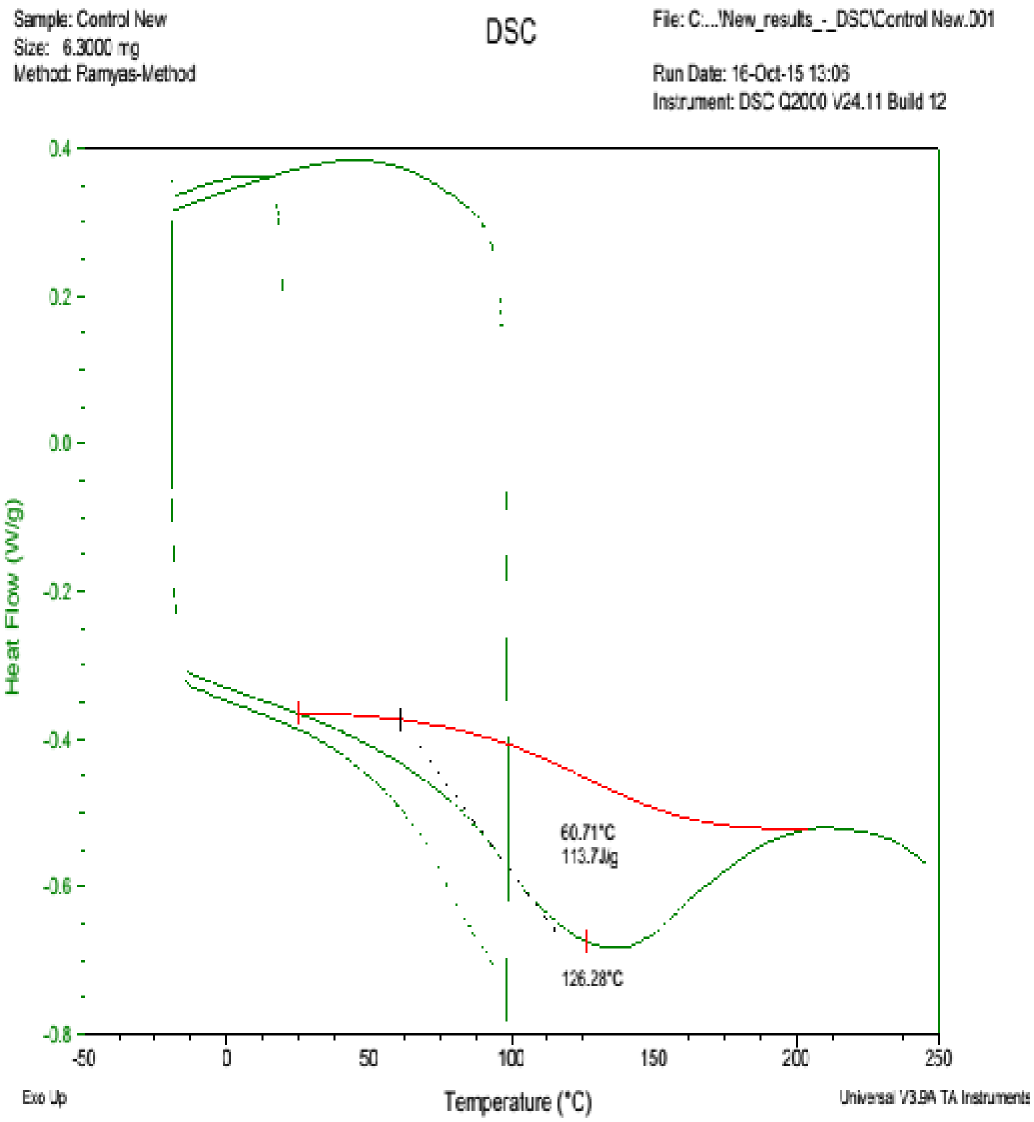
(Glycyrrhiza glabra)

Figure 62



Differential Scanning Calorimetric analysis of 0.1 per cent AgNP *(Glycyrrhiza glabra)*

Figure 63



Differential Scanning Calorimetric analysis of Control (Tapioca Starch)

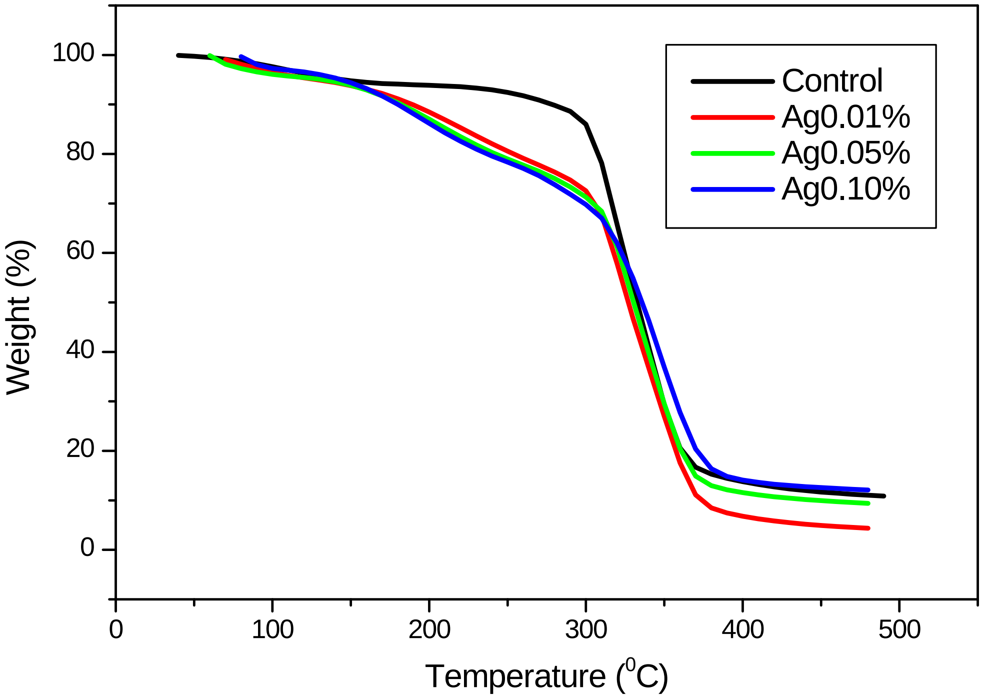
Figure 64

Increase in silver concentration in the edible film shows a melting enthalpy of the edible film of 0.01, 0.05 and 0.1 per cent Ag indicating a crystalline nature (59.06, 27.50, 71.88 (ΔH) J/g respectively), when compared to enthalpy of 113.70 (ΔH) J/g of the control (tapioca starch). Reduction in enthalpy is observed, compared to the control. However, the silver concentration in the films do not have straight correspondence. There is no change in glass transition temperature (T_g) corresponding to the concentration of Ag nanoparticles impregnated starch film. The effect of hinderance may also be due to the ability to form thetriple helical structure, resulting in decreased degree of heat of fusion and crystallinity in film. Hassan *et al.,* (2012) report similar results and provide various causes to explain the phenomenon.

Figures 61 to 64 depicts that a broad peak is observed near 111.33°C, which may be attributed to the melting of 0.01 per cent Ag incorporated film. It is interesting that the melting point of 0.05 and 0.1 per cent Ag impregnated film (T_m)displays a broad melting endothermic peak and shifts to a lesser temperature say 93.45ºC and 117.12ºC respectively, compared to the tapioca starch film (control) at 126.28 ºC. Hence, in our study, melting temperature (T_m) is reduced with an increase in Ag concentration of film which may be due to the reduced size and shape of the silver nanoparticles. Arvanitoyannis *et al.,* (1997) report that the reduction in melting point (T_m) of Ag nanoparticles incorporated film could be due to their larger surface area to volume ratio of nanoparticles which alters their thermal and thermo dynamic properties. Therefore, the discrepancy in the thermal data may be correlated to the size distribution of the particles probably. In the present study, it is also observed that the melting temperature (T_m) of starch impregnated with Ag nanoparticle film has shifted to lower temperature, when the nanoparticle concentration is increased from 0.01 to 0.1 per cent Ag film. The reduction in melting temperature may be attributed to the interaction of glycerol between the starch and nanoparticles forming hydrogen bonds, thereby producing a lower energiesin the edible film.

iv. Thermo Gravimetric Analysis (TGA)

Figure 65 depicts the Thermo Gravimetric analysis (TGA) of edible film of varied concentration of edible film at 0.01, 0.05 and 0.1 per cent Ag and control.



Thermo Gravimetric Analysis of Silver Nanoparticles
(Glycyrrhiza glabra)

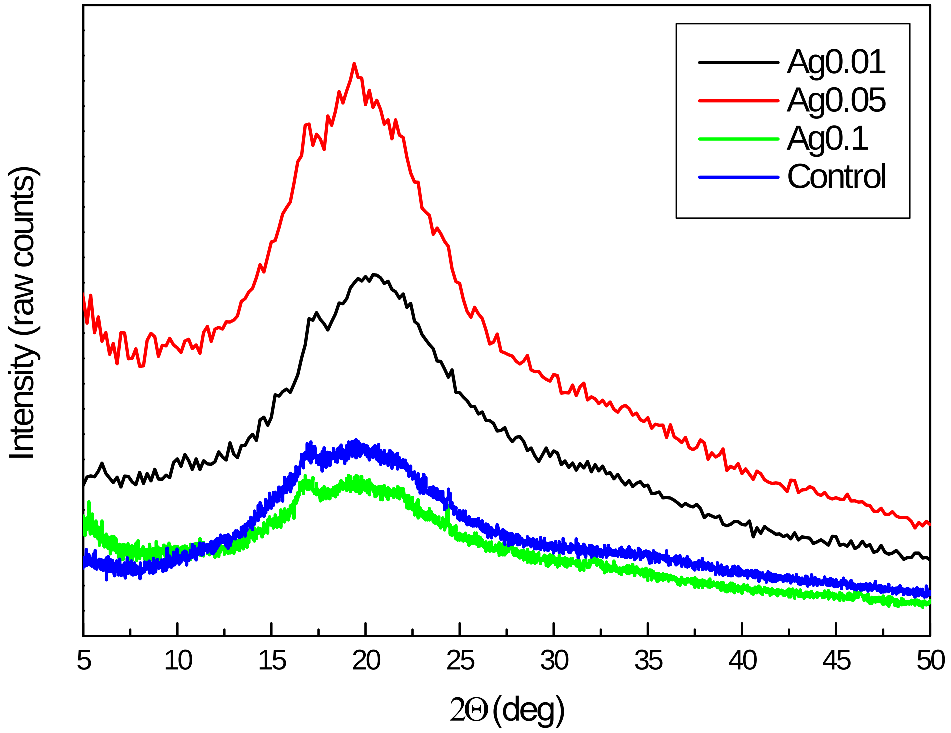
Figure 65

Thermal gravimetric analysis(TGA) is employed to determine the thermal decomposition and stability of silver nanoparticle impregnated edible films (0.01, 0.05 and 0.1 per cent) and control (tapioca starch) are obtained by heating at a rate of 20 °C /min between 20°C and 500°C. The TGA provides the information on the thermal behavior of edible film such as pyrolysis and decomposition of the edible film. The relationship between the weight loss and temperature are shown in Figure 65. Thermal analysis showed that the films are stable up to 90ºC for all the concentrations of Ag nanoparticle impregnated edible film. The TGA curve reveals three significant thermal events (Figure 84). The first region between the temperature 90–100 ºC is attributed to the vaporization of absorbed surface and evaporation of bound water and the second region between 100-300 ºC is due to the degradation of glycerol in the edible film. The third region occurs between at 300-375 ºC is related to the degradation of the starch polymer. The onset of thermal decomposition of starches occurs at 375 ºC for the edible films. Due to the incorporation of silver nanoparticle at three different concentrations of 0.01, 0.05 and 0.1 per cent, the degradation preceeded 360ºC respectively. These results prove that as the silver nanoparticles concentration in film increased degradation temperature rises.

Among varied concentration of silver nanoparticles impregnated edible film, the TGA results of 0.01 per cent edible film was found to be the best film. It exhibited degradation at 370ºC. The tapioca starch (control) degraded at 380ºC. The degradation temperature of silver nanoparticles impregnated edible film of (0.05 and 0.1) are390ºC. The pyrolysis of these Ag impregnated edible film takes place between 300 ºC to 400 ºC respectively. Khanna *et al.,* (2005) stated that these degradation events may be due to loss of functional groups, back bone fragments and get finally charred. Thus, the initial thermal decomposition temperature is shifted to a higher value by increasing the silver nanoparticle concentration in the Ag impregnated tapioca starch film. Hence, in the present study, the incorporation of silver impregnated edible films improved the thermal stability 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).

v. X-Ray Diffraction (XRD)

Figure 66 depicts the XRD of edible film at three different concentration of 0.01, 0.05, 0.1 per cent Ag and control (tapioca starch).



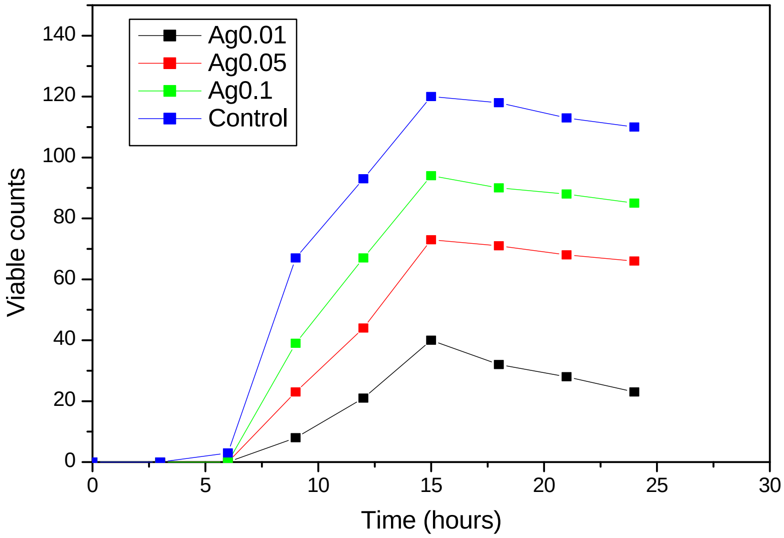
X Ray Diffractogram Pattern of Edible Films
(Glycyrrhiza glabra)

Figure 66

Figure 66 shows the XRD diffractogram of all the films prepared. The peaks are not prominent due to the screening effect of starch over Ag particles. However, the peaks at 11 and 19 (degree) (2θ) show that the characteristic planes namely 38 and 40. This is a definite confirmation for the presence of Ag in the films. The crystallinity is observed to increase with the concentration of silver. The presence of amylose is confirmed in all the films of 0.01, 0.05 and 0.1 per cent Ag impregnated tapioca starch film with characteristic peaks at 2θ =18° and 20° corresponding 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 with characteristic peaks at 2θ =16° and 19° confirming the amorphous nature with 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.

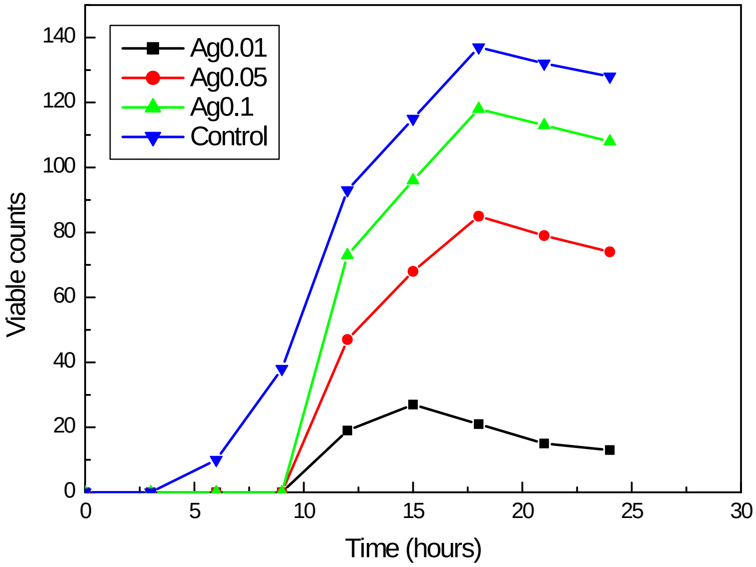
vi. Antimicrobial efficiency of Edible films against *EnteropathogenicSpecies*

Population viability of *Escherichia coli* in control plates (tapioca starch) and in petrid plates coated with the different films of 0.01, 0.05 and 0.1 per cent Ag nanoparticles impregnated tapioca starch are shown in Figure 67, 68 and 69.



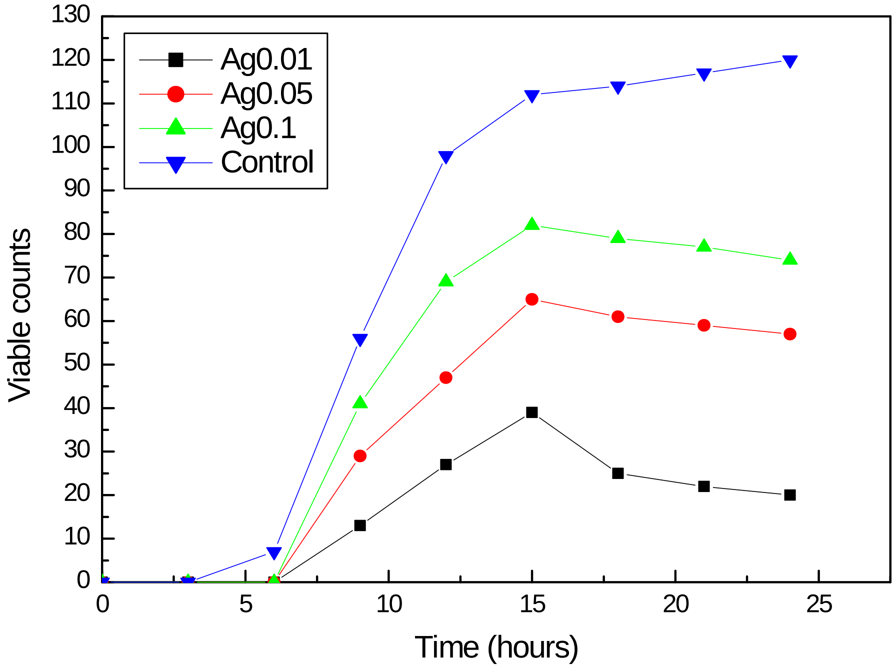
E.Coli **Growth Curve using Silver Nanoparticle Edible Films**

Figure 67



S.enterica **Growth Curve using Silver Nanoparticle Edible Films**

Figure 68



***Sh. dysenteriae* Growth Curve using Silver Nanoparticle Edible Films**

Figure 69

From 0 to 3 hours, there was significant antimicrobial activity among the three films of Ag impregnated nanofilms, whereas the bacterial growth was observed in control plates of three viable counts of *E.coli* at 6th hour at incubation period of 37 °C (tapioca starch film without Ag nanoparticles). During the 9th hour of incubation, that the 0.01, 0.05 and 0.1% Ag impregnated nanofilms had 88.06, 65.67 and 41.79 per cent respectively of *E.coli* inhibition against the control (tapioca starch). In the 15th hour, the Ag impregnated film of 0.01, 0.05 and 0.1% showed *E.coli* inhibition of 66.67, 39.17 and 21.67 per cent against the control. From the 12th to 15th hour, there was a decline in *E.coli* viable counts among all the three edible films of 0.01, 0.05 and 0.1 per cent Ag impregnated film may be due to lag phase of *E.coli* growth. From 18th to 21st hour, the Ag impregnated films of 0.01 per cent showed the *E.coli* inhibition ranging from 72.88 to 75.22. However, 0.05 and 0.1 per cent Ag impregnated edible film showed microbial inhibition of 39.83 to 39.82 and 31.11 to 22.12 per cent respectively. The viable counts of*E.coli* werefound to be stagnant from 18th to 21st hour which may be due to the stationary phase of *E.coli* growth. During 24th hour, the Ag impregnated film of 0.01, 0.05 and 0.1 per cent showed *E.coli* inhibition of 79.09, 40 and 22.72 per cent respectively against the control.

Among the three different concentrations of 0.01, 0.05 and 0.1 per cent Ag impregnated nanofilm,it was observed that 0.01 per cent Ag film had the highest antimicrobial activity against *E.coli.* The strong antibacterial property of 0.01 per cent Ag impregnated film may due to:

i. The smaller particle size of 34.7 nm with a high zeta potential of -28.9 mV and increased stability helps to act against the diarrheal species of *E.coli*

ii. Antibacterial mechanism of 0.01 per cent Ag impregnated films may be due to the dose of Ag nanoparticles from the edible film, which deeply penetrates inside the bacterial cell wall to modulate cellular signaling by dephosphorylating the peptide on tyrosine residues.

iii. Antibacterial activity of Ag impregnated film also be due to the AgNPs which are shape dependent particles that interact with *E.coli*.

iv. The lattice plane of the truncated triangular silver nanoplates has the strongest biocidal action, as observed from Figure 53 and 54 (SEM and TEM) from phase I, when compared to rod shaped and spherical nanoparticles

v. The mechanism of *E.coli* inhibition could be due to the Ag nanooparticle that induce the toxicity to bacterial cells by producing reactive oxygen species (ROS), reduction in GSH, elevating ROS levels, lipid peroxidation DNA damage and necrosis of the bacterial cell

vi. Siva Kumar *et al.*, (2013) proposed the nanocomposite film based on starch PVA blends.The antibacterial activity of Ag impregnated film may be due to the attachment of silver colloids attacking the cell membrane and causing accimilization of protein precursors leading to the dissipation of protein.

It also exhibited the destabilization and rupture of the plasma membrane, thereby causing depletion of intracellular ATP. The nano silver reacts with oxygen and sulphydryl (SH) groups on the cell membrane forming R-S-S-R bonds , thereby inhibiting the microbial respiration, resulting in cell death.

The antibacterial effect of the three Ag impregnated nanofilms of 0.01, 0.05 and 0.1 per cent coated onto petrid plates and the control petrid plates (tapioca starch) were tested against *Salmonella enterica (MTCC 3219)* and its cell viability is depicted in Figure 87. It is observed that from 0 to 3 hours, there was no growth of viable cells of S.enterica (MTCC 3219). In the 10th hour, the Ag impregnated nanofilms of 0.01, 0.05 and 0.1% Ag did not have any viable cell count of *S.enterica (MTCC 3219)*, but the control (tapioca starch) had 10 viable cell counts of *S.enterica (MTCC 3219).* During the 12th hour, Ag impregnated nanofilm of 0.01, 0.05 and 0.1 per cent Ag showed microbial inhibition of 79.57, 49.46 and 21.50 per cent respectively. From the 15th to 18th hour, *S.enterica (MTCC 3219)*inhibition *of* 0.01, 0.05 and 0.1 per cent Ag film are from 76.52 to 84.67 per cent, 40.87 to 37.96 per cent and 16.52 to 13.87 per cent respectively. From the 18th to 24th hour, there was an increment in microbial inhibition of all the three nanofilms of 0.01, 0.05 and 0.1 per cent. The quantity of Ag was 84.67 to 89.84 per cent, 37.96 to 42.19 per cent and 13.87 to 15.62 per cent respectively. From the present antibacterial study, it is observed that of the three Ag impregnated nanofilms, 0.01 per cent Ag showed the maximum microbial inhibition against *S.enterica (MTCC 3219)* than 0.05, 0.1 per cent Ag impregnated filmand control (tapioca starch) which had less microbial inhibition against *S.enterica (MTCC 3219)*of all the diarrheal species in the study.

However, due to the presence of antioxidants such as thiol and phenolic functional groups (as observed in phase I).TLC and FTIR analysis of the Ag impregnated film showed the highest antibacterial activity against the diarrheal species of *E.coli.*The most abundant antioxidant compounds found in *Glycyrrhiza glabra Ag impregnated nanofilms are* 9Octadecenal (RT=14.75) is an aldehyde compound, cis - 9,10 Epoxyoctadecan-1-ol (RT =17.34) and Z,Z,2,5-Pentadecadien-1-ol (RT=20.71) are the two major alcoholic compounds which possess the antimicrobial property. Among the esters and organic acids, the most dominant fatty acids are tetradecanoic acids (RT=11.58), n hexadecanoic acid (RT=12.84) which possess the antioxidant property of scavenging the free radicals. In addition, the antibacterial mechanism of Ag impregnated film may be due to both the antioxidants and Ag nanoparticles, which induces the formation of superoxide radicals that attack the target sites of fatty membrane and initiates lipid peroxidation, As a result, the membrane properties are changed because of insufficient membrane fluidity leading to disruption of membrane bound proteins. Further, the antimicrobial activity may be due to antioxidants and nanoparticles, which target the DNA, in which the ROS attack both the nitrogen base and sugar, resulting in single and double strand breaks in the back bone. The ROS manifests its toxicity by oxidation of sulfhydryl groups, oxidation of aminoacid, modificiation of prosthetic groups and peptide fragmentation of bacterial cells. These reactions affect the functions of membrane proteins blocking DNA replication. Stadtman (1990) propounds similar findings on antibacterial mechanism of silver nanoparticles.

The viable cell of *S.dysenteriae* species were obtained from the PSGIMS, Coimbatore. The antibacterial activity of Ag impregnated film of 0.01, 0.05 and 0.1 per cent Ag were tested against the *S.dysenteriae (PSGIMS&R)* and are shown in Figure 67, 68 and 69. From 0 to 3 hours, there was no viable cell growth of all the three Ag impregnated nanofilm of 0.01, 0.05 and 0.1 per cent Ag. During the 6th hour, there was no viable cell growth of *Sh.dysenteriae* (PSGIMS&R), but only the control (tapioca starch) had 7 microbial counts of *Sh.dysenteriae (PSIMS&R).* In the 12th hour, the Ag impregnated nanofilm of 0.01, 0.05 and 0.1 per cent Ag showed a maximum inhibition of 97.72, 52.04 and 29.59*Sh.dysenteriae (PSIMS&R)*respectively. In the 5th hour, there was a gradual decline in microbial inhibition with the counts of 65.18, 41.96 and 26.79 per cent respectively against the *Sh.dysenteriae (PSIMS&R).* From the 18th to 24th hour, it is observed that the Ag impregnated nanofilm of 0.01, 0.05 and 0.1 per cent Ag showed the inhibition of 78.07 to 83.33 per cent, 30.71 to 52.50 per cent and 30.7 to 38.33 per cent respectively against the live cells of *S.dysenteriae.* Hence, the present study, reveals that among the three Ag impregnated nanofilm, 0.01 per cent Ag showed a maximum microbial inhibition against the live cells of *S.dysenteriae,* compared to 0.05, 0.1 per cent Ag nanofilm and control (tapioca starch).

The antibacterial mechanism of Ag impregnated nanofilm synthesized from *Glycyrrhiza glabra* may be due to the phenolic and thiol groups which interact with ionic silver and inactivate the bacterial cell, when treated with Ag nanoparticles. In the present study, it was also reported that nanosilver causes the inactivation of bacteria, as these silver nanoparticles interact with the bacterial chemical groups that drag the sulphur or halide groups. In addition, gram negative bacteria were highly sensitive to silver than gram positive bacteria, which is due to the presence of the negatively charged lipopolysaccharide (LPS) that attract the positively charged silver ions, thereby killing the bacterial cell..

The silver nanoparticles of silver cations interact with atoms with a high electron density possessing extreme chemical affinity for sulphur groups like thiol groups (-SH) in biomolecules. In addition, the interaction of silver with L-Cysteine residues caused the denaturation and loss of enzymatic functions. Another possible antibacterial mechanism could be due to the inactivation of enzymes in the outer membrane, permeability and energy metabolism of bacterial cell disruption, leading to a loss in proton motive force. Due to electrolyte imbalance, a massive loss of potassium may also be possible. In addition, entry of Ag ions into the cell inhibit dehydrogenase activity of the respiratory chain leading to depletion of intracellular ATP. These silver ions also intercalate between N-H bonds in purine and pyrimidine residues. Hence, these ions inhibit the cell replication of bacterial cell. Therefore, Ag impregnated edible film can act as an antimicrobial agent against the diarrheal pathogens.

The inhibitory effect of these Ag impregnated nanofilms modulatethe phosphotyrosine proteins and arrests its bacterial growth. Thus, Ag impregnated nanofilms are found to be 16.7 mg/kg below the requirements of Overall Migration Limit (OML). Therefore, these nanofilms are more flexible as an antimicrobial wrapping in food packages. The OML of Ag is 60 mg of substances/kg (of food stimulant) for all substances has maximum migration limits with the regulations of No. 10/2011.