3.1. SYNTHESIS OF NANOPARTICLES

3.1.1. SYNTHESIS OF PLA-PEG NANOPARTICLES

Poly lactic acid – poly ethylene glycol (PLA-PEG) nanoparticles were synthesised through double emulsion solvent evaporation method as reported by Mainardes et al. (2010). Different quantities of polylactic acid (PLA) and polyethylene glycol (PEG) were dissolved in 2 ml dichloro methane (DCM) and mixed with 200 µl polyvinyl alcohol (PVA, 0.1% w/v) containing green tea polyphenols (GTP). The mixture was sonicated for 30 s at 55 W to form w/o emulsion. About 4 ml of PVA (0.7% w/v) was added and sonicated for 1 min at 55 W to form w/o/w emulsion. Organic solvent was evaporated at room temperature. Particles were recovered by centrifugation and washing twice with milli Q water. Three different combinations of PLA-PEG i.e. 1:3, 1:1 and 3:1 and GTP loading concentrations in a range of 1 to 15 mg/ml were used.

Yield of GTP loaded PLA-PEG nanoparticles were calculated using the following formula:

\[
\text{Yield} \, (\%) = \frac{(\text{Weight of polymer} + \text{GTP}) - \text{Weight of nanoparticles formed}}{(\text{Weight of polymer} + \text{GTP})} \times 100
\]

3.1.2. SYNTHESIS OF LIPOSOMAL NANOPARTICLES

Phosphatidyl choline and cholesterol were taken in 20:1 ratio and dissolved in 2 ml ethanol to form a lipid phase. This mixture was heated in a water bath at 60°C. About 0.1% Tween 80 was dissolved in 10 ml of phosphate buffer saline (PBS) (pH 6.5) to form an aqueous phase. Aqueous phase obtained was then added to the lipid phase (phosphatidyl choline and cholesterol dissolved in ethanol) upon stirring. The mixture was placed on a magnetic stirrer for 10 min followed by sonication at 30% amplification for 10 min. Final liposomal nanoparticles were obtained after being cooled in an ice bath and diluted to 20 ml and filtered through Whatmann filter paper No1. For GTP loaded liposomal nanoparticles, different concentrations of GTP (1,
2.5, 5 and 10 mg/ml) were dissolved in ethanol prior to the dissolution of phosphotidyl choline and cholesterol (Guan et al., 2011).

3.1.3. SYNTHESIS OF CASEIN NANOPARTICLES
Casein nanoparticles were synthesised by cross linking the nanoparticles with glutaraldehyde using desolvation technique. About 200 mg of casein was dissolved in 2 ml of 1N NaOH. The solution was stirred continuously and 8 ml of ethanol was added drop wise at a constant rate of 1 ml/min which resulted in the formation of casein nanoparticles. The particles formed were stabilized by addition of 8% glutaraldehyde (1.175 μl/mg casein). The cross linking was performed for about 24 h at room temperature under constant stirring in a magnetic stirrer (Weber et al., 2000). For preparation of GTP loaded casein nanoparticles, 1 to 10 mg/ml GTP was added to the reaction mixture before cross linking, followed by addition of glutaraldehyde. The mixture was centrifuged at 8000 rpm for 20 min at 4°C. The pellet contained nanoparticles while the supernatant was used for quantification of unloaded GTP.

3.1.4. SYNTHESIS OF ALBUMIN NANOPARTICLES
GTP free albumin nanoparticles and GTP loaded nanoparticles were synthesised by cross linking the particles with glutaraldehyde using desolvation technique (Weber et al., 2000; Langer et al., 2003). About 10% albumin suspension was prepared in water. The solution was stirred continuously and 8 ml of ethanol was added drop wise at a constant rate of 1 ml/min which led to the formation of albumin nanoparticles. The particles formed were stabilized by the addition of 8% glutaraldehyde (1.175 μl/mg albumin). The cross linking was performed for about 24 h at room temperature under constant stirring. For preparation of GTP loaded albumin nanoparticles, 1 to 15 mg of GTP was added prior to cross linking, followed by addition of glutaraldehyde (Table 6.1). To optimize the drug loading efficiency, different parameters like volume of ethanol (3, 8 and 10 ml), percentage of glutaraldehyde (3, 8 and 10%) and pH of the dissolution medium of albumin (3, 6.5 and 9 pH) were varied. Drug (GTP) loading efficiency was determined by spectrophotometrically measuring the amount of free drug in the supernatant and calculating the percentage of drug loaded into the nanoparticles.
3.2. PURIFICATION OF NANOPARTICLES
Nanoparticles formed was purified by three rounds of centrifugation (8000 rpm, 20 min) followed by redispersing of pellet in equal volume of water. For each redispersion, ultrasonication was carried out for 5 min.

3.3. CHARACTERIZATION OF NANOPARTICLES

3.3.1 PARTICLE SIZE AND ZETA POTENTIAL ANALYSIS
Size of the nanoparticles and the surface charge (zeta potential) of the particles were measured through dynamic light scattering (DLS) and zeta potential analysis. Particles were dispersed in water prior to measurement. The analysis was carried out using Nanopartica, Nanoparticle analyzer SZ-100.

3.3.2. ATOMIC FORCE MICROSCOPY (AFM)
Morphology, polydispersity and particle size of casein nanoparticles were analyzed by atomic force microscopy. The sample preparation was carried out using 100 µl of nanoparticulate suspension, mixed with ethanol, followed by making a thin smear on a clean glass slide and allowed to dry. The images were captured in AFM (Nano Surf Easy Scan2, Switzerland).

3.3.3. HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY (HR SEM)
Morphology and polydispersity of synthesized nanoparticles were analyzed by scanning electron microscope. About 50 µl of nanoparticle suspension was smeared on a carbon coated button, followed by gold sputtering. The images were taken in FEI Quanta FEG 200 – High Resolution Scanning Electron Microscope.

3.3.4. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)
The purified powder of nanoparticles was analysed through FTIR spectroscopy. The measurements were performed in Siemen 578564 instrument in the diffuse reflectance mode with a resolution of 4 cm\(^{-1}\) in KBr pellets. The pellets were later analysed through FTIR spectroscopic measurements.
3.3.5. X-RAY DIFFRACTION (XRD) CRYSTALLOGRAPHY
XRD analysis of prepared sample was performed using powder X-ray diffractometer (D8 Advance), BRUKER, Germany using 2.2 KW Cu anode, ceramic X-ray tube of wavelength (λ) 1.54056 Å° and angle range from 10° to 80° with a step of 0.02°.

3.3.6. UV-VIS ABSORPTION STUDIES
UV-visible absorption studies of prepared sample were carried out in Lasany double beam 1-2902, UV-vis spectrophotometer in a wavelength range of 200-500 nm. A quartz cuvette (4 x 1 x 1 cm) with 1 cm path length was used. All the solutions used for measurement were prepared in double distilled water.

3.3.7. CIRCULAR DICHROISM (CD) SPECTROPOLARIMETRY
The spectra of prepared sample was recorded using Jasco, J-715 CD spectrometer. The measurements were carried out in a quartz cuvette with 1cm path length at a wavelength range of 200-290 nm with a bandwidth of 1 nm.

3.3.8. FLUORESCENCE QUenching MEASUREMENTS
The fluorescence measurement of prepared sample was recorded in Jasco FP-8300 fluorescence spectrophotometer. The excitation wavelength was set at 278 nm and the emission was measured in a range of 275-475 nm with fixed emission slit width of 10 nm. The measurements were carried out in a quartz cuvette with a path length of 1 cm.

3.4. MOLECULAR DOCKING STUDIES
Constituents of GTP [(−)-Epigallocatechin-3-gallate (EGCG), (−)-Epicatechin-3-gallate (ECG), (−)-Epigallocatechin (EGC), (−)-Epicatechin (EC) and (+) catechin] were docked with the target protein, bovine serum albumin (BSA) to confirm its binding potential. The docking analysis was performed through Auto dock tools (ADT) v1.5.4 and Autodock v4.2 programs. The chemical structures of constituents of GTP (ligand) were taken from the PubChem Compound database (http://www.ncbi.nlm.nih.gov/search). Three-dimensional structures of the target protein, BSA (ID: 4L9Q) was retrieved from the Protein Data Bank (PDB) (http://www.pdb.org). The active sites of the preferred target proteins were searched.
using Q-site Finder, where a putative ligand could bind and optimized its van der Waals interaction energy (Laurie and Jackson., 2005). The grid box was centered with x-, y-, and z-axes (40.862, −0.511, and 12.219, respectively) with a spacing of 0.375 Å. Constituents of GTP were docked to target protein complexes with the molecule considered as a rigid body and the ligands being flexible. The search was extended over the whole receptor protein used as blind docking.

3.5. DETERMINATION OF LOADING EFFICIENCY (LE)

Estimation of GTP for calculating the loading efficiency was carried out by Folin-Ciocalteu assay as described by Swain and Hillis (1959). Briefly, 0.5 ml of sample was added to 0.5 ml Folin - Ciocalteu reagent (1M) and 0.5 ml sodium carbonate (35%) and incubated in dark for 30 min. The absorbance was recorded at 700 nm in a spectrophotometer. The concentration of GTP was calculated from standard curve of GTP. All experiments were carried out in triplicates. The loading efficiency (LE) was calculated using the following formula:

\[
\text{LE} \, \% = \frac{(\text{Amount of GTP} - \text{Amount of free GTP})}{\text{Total amount of GTP}} \times 100
\]

3.6. IN VITRO RELEASE PROFILE OF GTP LOADED NANOPARTICLES

Release studies were carried out through dialysis method. About 1 ml of GTP loaded PLA-PEG nanoparticles were loaded in dialysis membrane (having a molecular weight cut off value of 3.5 kDa) with both ends sealed. The dialysis bag was then placed in a beaker with 10 ml PBS at pH 7.4. At different time intervals, 1 ml from the beaker was collected, which was replaced by the equal volumes of fresh PBS. The samples were centrifuged and the supernatant was used to determine the amount of GTP released. Samples were collected from 1 to 48 h at regular time intervals. The estimation of GTP was carried out by the method of Swain and Hillis (1959) as described in section 3.5. Release studies was carried out for different ratios of PLA-PEG, liposomes, casein and albumin nanoparticles for different loading concentrations of GTP and by varying the release conditions such as pH (3, 7.4 and 9) and temperatures (room temperature, 37 and 45°C).
3.7. EFFECT OF ALCOHOL ON RELEASE OF GTP FROM GTP LOADED CASEIN AND ALBUMIN NANOPARTICLES

About 1ml of GTP loaded casein and albumin nanoparticles was placed in a beaker with 10 ml release medium of pH 7.4 and incubated at 37°C. The release medium contained either PBS alone or along with 10, 20, 30 or 40% ethanol. About 1ml of sample was withdrawn at different time intervals as mentioned in section 5.1.4. The collected samples were centrifuged and the supernatants were used for estimation of released GTP as described in section 3.5.

The drug release profile of alcohol and non alcoholic media was compared using $f_2$ similarity factor according to the expression given by Moore and Flanner (1996). Similarity factor was determined as per the following formula:

$$f_2 = 50 \times \log \left\{ \left[ 1 + \frac{1}{n} \sum_{t=1}^{n} (R_t - T_t)^2 \right]^{-\frac{1}{2}} \right\} \times 100$$

Where $n$ is the number of dissolution time points, $R_t$ and $T_t$ are the percentage drug dissolved of reference (non alcoholic medium) and test (alcoholic medium) at time point $t$.

Relative change in the amount of dissolution in alcoholic medium compared to non alcoholic medium was calculated as per the following equation as described by Smith et al (2010b). To determine the relative change in the amount of dissolution, the drug dissolution in alcoholic and non alcoholic medium was determined using the formula:

$$C_n = C_{std} \times \frac{Abs \ (sample)}{Abs \ (std)}$$

Where, $C_n$ is the sample concentration at time point $n$; $C_{std}$ is the concentration of the standard (mg/ml); $Abs \ (sample)$ is the absorbance of the sample solution and $Abs \ (std)$ is the absorbance of the standard solution.

The sample concentration obtained was then applied to calculate the percentage of drug dissolution using the following equation:

$$D_n = 100 \times [C_n \times (V - a \times (n - 1)) + a \times \sum C_n - 1]$$
Where Cn is the sample concentration at time point n; V is the initial vessel volume (ml); a is the aliquot of the sampling volume (ml); and n is the time point.

Therefore, the relative change in the amount of dissolution in alcoholic media compared to non alcoholic medium was calculated according to the following equation

\[ D_{A/N} = 100 \times \frac{D_A - D_N}{D_N} \]

Where, \( D_A \) is the percentage of GTP dissolved in alcoholic medium and \( D_N \) is the percentage of GTP dissolved in non alcoholic medium (PBS).

3.7.1. SWELLING BEHAVIOUR

Swelling experiments were conducted in non alcoholic and alcoholic media (PBS and PBS with 10, 20, 30 and 40% alcohol). GTP loaded albumin nanoparticles were weighed (\( w_0 \)). About 2 ml of media (PBS or PBS with 10, 20, 30 and 40% alcohol) was added and left for 120 min at 37°C. Samples were then removed, centrifuged and re weighed (\( w_1 \)). Percentage of swelling was calculated according to the following formula of Huang et al (2007):

\[ Swelling (\%) = \frac{w_1 - w_0}{w_0} \times 100 \]

3.7.2. MEDIA UPTAKE STUDIES

Media uptake of GTP loaded albumin nanoparticles was studied. About 0.1 gm of GTP loaded albumin nanoparticles was placed in a definite volume (2 ml) of medium (PBS or PBS with 10, 20, 30 and 40% alcohol). Media uptake studies were carried out for 120 min at 37°C. Nanoparticles were separated by centrifugation (4000 rpm for 10 min) and weighed (\( w_0 \)). The samples were dried in an oven for 15 min at 37°C and again re weighed (\( w_1 \)). Media uptake capacity was determined using the following formula (Kreye et al., 2011).

\[ Media \ uptake \ capacity \ (\%) = \frac{w_0 - w_1}{w_0} \times 100 \]
3.7.3. WETTABILITY/CONTACT ANGLE MEASUREMENT
Contact angle measurements were studied to determine the wettability of GTP loaded albumin nanoparticles. Contact angles of nanoparticles with dissolution media of PBS and PBS with 10, 20, 30 and 40% alcohol were examined through drop shape method (Buvailo et al., 2010). The measurements were carried out by placing a drop of sample on a glass slide. The contact angle was observed visually by observing the shape of the drop (< 90°, 90° and > 90°). The experiment was carried out in triplicates.

3.8. RELEASE OF GTP IN SIMULATED BIOLOGICAL FLUIDS
In vitro release of GTP was also performed in simulated conditions of saliva (SSF, pH 6.5) [Potassium chloride (0.72 g/L); Calcium chloride dihydrate (0.22 g/L); Sodium chloride (0.6 g/L); Potassium phosphate monobasic (0.68 g/L); Sodium phosphate dibasic (0.86 g/L); Potassium bicarbonate (1.50 g/L); Potassium thiocyanate (0.06 g/L); Citric acid (0.03 g/L)], gastric fluid (SGF) (pH 1.2) [Sodium chloride (0.2% w/v); Pepsin (0.1 mg/ml); Hydrochloric acid (0.7% v/v)], intestinal fluid fasted state (SIF) (pH 6.5) [Sodium taurocholate (3 mM); Lecithin (0.75 mM); Sodium hydroxide (0.34 g/L); Sodium phosphate dibasic (3.94 g/L); Sodium chloride (6.18 g/L)]; intestinal fluid fed state (pH 5) [Sodium taurocholate (15 mM); Lecithin (3.75 mM); Sodium hydroxide (4.04 g/L); Glacial acetic acid (8.65 g/L); Sodium chloride (11.87 g/L)] and colon fluid (SCF), (pH 7) [Potassium chloride (0.2 g/L); Sodium chloride (8.0 g/L); Potassium phosphate monobasic (0.24 g/L); Sodium phosphate (1.44 g/L)] in both fasted and fed states following the guidelines of US Pharmacopeia (Marques 2004; Marques et al., 2011). Release of GTP was carried out in SSF, SGF, SIF and SCF for 2 min, 2 h, 6 h and 24 h respectively simulating the process of digestion as the food passes through the saliva, stomach, intestine and colon. The release studies were carried out through dialysis membrane (having a molecular weight cut off value of 3.5 kDa) and the samples were withdrawn from the respective fluid at the respective time.

3.9. MATHEMATICAL MODELLING AND RELEASE KINETICS
To study the drug release mechanism, in vitro release data were fitted into different mathematical models like Zero order, First order, Higuchi, Hixson–Crowell and Korsemeyer-Peppas models (Costa and Lobo., 2001). The best fit of the models was
determined by analysing the correlation coefficient ($R^2$) value and release exponent ($n$) to determine the mechanism of release (Table 3.1).

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>Parameter’s definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero order</td>
<td>$C = k_o t$</td>
<td>$C$ - Concentration of drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$k_o$ - Zero order constant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t$ - Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$C_o$ - Initial concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$C_t$ - Concentration at time $t$</td>
</tr>
<tr>
<td>First order</td>
<td>$\log C_o - \log C_t = k_1 t / 2.303$</td>
<td>$k_1$ - First order constant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t$ - Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Q$ - Amount of drug released in time $t$</td>
</tr>
<tr>
<td>Higuchi Model</td>
<td>$Q = k_H t^{1/2}$</td>
<td>$k_H$ - Higuchi constant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Q_o$ - Initial amount of drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Q_t$ - Remaining amount of drug at time $t$</td>
</tr>
<tr>
<td>Hixson Crowell Model</td>
<td>$Q_o^{1/3} - Q_t^{1/3} = k_{HC} t$</td>
<td>$k_{HC}$ - Hixson Crowell constant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t$ - Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$M_t - M_o$ - Fraction of drug released at time $t$</td>
</tr>
<tr>
<td>Korsmeyer-Peppas Model</td>
<td>$M_t - M_o = kt^n$</td>
<td>$k$ - Korsmeyer-Peppas constant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t$ - Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$n$ - Release exponent</td>
</tr>
</tbody>
</table>

3.10. DETERMINATION OF ANTIOXIDANT PROPERTY OF RELEASED GTP

Antioxidant activity of released GTP was determined in vitro by 1,1-diphenyl-2-picyrylhydrazyl (DPPH) assay following the method reported by Williams et al (1995). DPPH was prepared in absolute ethanol at a concentration of 0.1mM. About 100 μl sample was added to 550 μl PBS (pH 7.4) and 0.5 ml of DPPH. The reaction
constituents were incubated for 30 min at room temperature in dark and absorbance was recorded at 517 nm in UV-visible spectrophotometer against control solution (without the sample). The experiment was repeated thrice.

3.11. IN VITRO CYTOTOXICITY STUDIES

*In vitro* cytotoxicity of albumin and GTP loaded albumin nanoparticles were studied against cancerous cells and normal cells through MTT assay. Briefly, the MCF7 breast cancer cells and PC3 prostate cancer cells and 3T3 normal cells [procured from National Center for Cell Science (NCCS), Pune, India] were cultured in DMEM (MCF7 and 3T3) and RPMI (PC3) medium respectively containing 10% FBS in a culture flask at 37°C and 5% carbon dioxide. On reaching confluency, the cells were plated in a 96 well plate with a cell concentration of approximately 5 x 10^3 cells/well and incubated for 24 h. Medium was removed and washed with PBS. To the control plates, serum free medium was added and to the treatment plates, different concentrations of free GTP, GTP loaded albumin nanoparticles and unloaded albumin nanoparticles were added at a concentration of 25, 50, 75 and 100 µg/ml. The medium was removed after 24 h of incubation and to remove the traces of drug, cells were washed with PBS twice, followed by the addition of fresh medium. About 5 µl of MTT solution (10 mg/ml concentration) was added to each well and incubated for 4 h. Upon removal of the medium, 100 µl DMSO was added to each well. Absorbance was recorded at 570 nm in an ELISA micro plate reader. The data are presented as percentage viable cells of the test group in comparison with the untreated control group.

3.12. BIOAVAILABILITY AND PHARMACOKINETICS IN IN VIVO (RABBIT MODEL)

Bioavailability and pharmacokinetics were studied in healthy New Zealand rabbits, weighing ~1.25 to 1.5 kg. Animals were procured from the central animal house of the Institute and the study protocol was approved by the institutional animal ethical committee (Approval No.: VIT/IAEC/VIII/24) for this experiment. The animals were kept in separate cages for about 45 days prior to the study for acclimatization. Throughout this period a constant day-night cycle was maintained and the temperature was set at 23±2°C. Before commencing the study, rabbits were fasted for
about 12 h with free access to water. On the day of study, free GTP and GTP loaded albumin nanoparticles were administered as a single dose of 50 mg/kg body weight orally.

Blood samples (0.5 ml each) were collected from the marginal ear vein using a butterfly infusion needle at different times (0.5, 1, 2, 4, 6, 8, 12 and 24 h) after the drug administration. Plasma was separated from the blood samples immediately by centrifugation at 2000 rpm for 15 mins and stored at -20°C till the assay was carried out. Plasma GTP concentration was determined through Folin-Ciocalteu assay method as carried out for in vitro release studies described in section 3.5 (Swain and Hillis., 1959).

3.12.1. PHARMACOKINETICS
The plasma concentration versus time data was analysed by open one compartment model. The kinetic parameters such as C_{max} (peak plasma concentration), T_{max} (time to peak plasma concentration), t_{1/2} (absorption and elimination half life), and AUC_{0-∞} (area under plasma concentration time curve) by trapezoidal rule were calculated. The ratio of individual AUC\_{0-∞} values of GTP loaded albumin nanoparticles and free GTP (relative bioavailability) were determined to access the extent of absorption from each formulation. The volume of distribution (V_{d}) was determined by considering the administered drug dosage and the initial plasma concentration. V_{d} was calculated using the following equation

\[ V_d = \frac{X}{C_p} \]

Where X is the administered dose and C_{p} is the initial plasma drug concentration. The data obtained from calculating V_{d}, drug clearance rate was calculated according to the following formula

\[ Cl = V_d \times K_{el} \]

Where V_{d} is the volume of distribution and K_{el} is the elimination rate constant. The mean residence time (MRT), corresponding to the drug transit time was calculated according to the following equation (Attia et al., 2007):

\[ MRT = \frac{1}{k_a} + \frac{1}{k_{el}} \]

Where, k_{a} is the absorption rate constant and k_{el} is the elimination rate constant.
3.13. TUMOR INDUCTION AND TREATMENT (IN MICE MODEL) – EVALUATION OF BIOCHEMICAL PARAMETERS

Five to six weeks old Swiss albino mice, weighing around 25-30 gm were selected for this study. They were procured from Institute’s animal house and the study protocol was approved by the institutional animal ethical committee (Approval No.: VIT/IAEC/VIII/24). The animals were divided into 6 groups, each group having 3 animals and were housed in standard polycarbonate cages under suitable laboratory conditions (standard conditions) at a temperature of 20°C and light of 12 h dark cycles with a humidity of 50-60%. Animals were fed with standard pellets and purified water. All the animals were acclimatized to the laboratory conditions before commencement of the experiment. Grouping of animals was as follows:

Group 1: Control (Normal mice)
Group 2: Tumor induced mice
Group 3: Mice pre-treated with free albumin nanoparticles before tumor induction
Group 4: Mice pre-treated with GTP loaded albumin nanoparticles before tumor induction
Group 5: Tumor induced mice treated with free albumin nanoparticles
Group 6: Tumor induced mice treated with GTP loaded albumin nanoparticles

Tumor induction of the Swiss albino mice were carried out at Amala Cancer Research Centre, Thrissur, India. Once the ascetic tumor was formed, transplantation to other mice was carried out in our laboratory. Ehrlich ascites carcinoma (EAC) cell line was used for tumor induction in mice. About $1 \times 10^6$ EAC cells were injected intraperitoneally to the mice. Treatment commenced after 10 days of tumor induction. Body weight of the mice was measured regularly. Free albumin nanoparticles and GTP loaded albumin nanoparticles were used as the test. Group 3 and 4 received 200 µl of free albumin nanoparticles and GTP loaded albumin nanoparticles respectively, while group 1 and 2 received 200 µl of PBS. Mice were administered 3 doses orally at an interval of 2 h and were starved throughout the experiment. The animals were sacrificed after the treatment. Plasma was separated from the blood samples immediately by centrifugation at 2000 rpm for 15 min and stored at -20°C along with the ascetic cells for further experiments.
3.13.1. EVALUATION OF BIOCHEMICAL PARAMETERS – ANTIOXIDANT ASSAYS

3.13.1.1. CATALASE

Catalase test was carried out in serum samples following Aebi’s method (1984). Briefly, 50 µl of sample was added to 1 ml of 30 mM hydrogen peroxide and 2 ml of phosphate buffer (pH 7.0). Spectroscopic measurements of the activity were carried out at 240 nm for 0 s, 30 s and 1 min. To measure the activity, molar extinction coefficient of H₂O₂ (43.6 Mcm⁻¹) was used. 1 mM of H₂O₂ degraded per minute is equal to one unit of catalase activity and expressed as unit per milligram of protein (Aebi, 1984).

3.13.1.2. SUPEROXIDE DISMUTASE (SOD)

This test was carried out following the method of McCord and Fridovich (1969). About 50 µl of plasma was added to 30 mM EDTA, 75 mM Tris HCl buffer (pH 8.2) and 2 mM pyrogallol. Spectroscopic measurements were carried out at 420 nm for 3 min. About 50% inhibition of the rate of auto oxidation of pyrogallol is considered as one unit of enzyme activity which was determined at 420 nm as change in absorbance per minute. The SOD activity is expressed as unit/mg protein.

3.13.1.3. LIPID PEROXIDATION (LPO)

Lipid peroxidation assay was carried out through thiobarbituric acid method as described by Ottolenghi (1959). About 50 µl of the plasma was added to 20% trichloro acetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was incubated for 10 min (in a boiling water bath) which was then centrifuged for 20 min at 3000 rpm after cooling. The supernatant’s absorbance was measured at 552 nm. The activity is expressed as unit/mg protein.

3.13.2. TUNEL ASSAY

Detection of apoptosis in ascetic cells collected from tumour induced mice was carried out through terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) assay using Trevigen TACS® 2 TdT-DAB in situ apoptosis detection kit. The assay was performed following manufacturer’s instruction. Briefly the method is as follows: Ascetic cells from all the groups were centrifuged at 500 g for 5 min. About 1x10⁶ cells were resuspended in 1 ml of 3.7%
buffered formaldehyde (fixative) and allowed to stand for 10 min at room temperature, followed by centrifugation for 5 min at 3000 rpm with the fixative being discarded and resuspending the cells in 80% ethanol. Cells were spotted on a clean glass slide and allowed to dry for 20 min at 45°C. Slides were then placed for 10 min in 70% ethanol and dried for 2 h at 45°C. Slides were then rehydrated in decreasing concentrations of ethanol (100, 95 and 70%) and finally washed in 1X PBS prior to labelling reaction.

Samples were flooded with 50 µl of Proteinase K and left at room temperature for 30 min and washed in deionized water twice. Slides were then placed in quenching solution for 5 min at room temperature and washed with 1X PBS for 1 min. Slides were then immersed for 5 min in 1X TdT labelling buffer. About 50 µl of labelling reaction mix was placed on the sample and incubated for 1 h at 37°C in a humid chamber. The labelling reaction was stopped by placing the samples in 1X TdT stop buffer in room temperature for 5 min. About 50 µl of Strep-HRP solution was added to the sample and incubated at 37°C for 10 min, followed by two washes for 2 min each in 1X PBS. Samples were then immersed in DAB solution for 5 min and washed in deionized water several times of 2 min duration. Methyl green was added to the samples and left for 3 min, followed by sequential washing in deionized water (10 times), 70% ethanol (2 changes), 95% ethanol (2 changes), 100% ethanol (2 changes) and xylene (2 changes). About 50 µl of mounting medium (glycerol) was added onto the sample and a cover slip was placed. Samples were stored in dark until observation under a microscope.

3.13.3. ENZYME LINKED IMMUNO SORBANT ASSAY (ELISA)
The expression levels of tumor necrosis factor α (TNF α), interleukin 2 (IL 2) and interleukin 10 (IL 10) was quantified through ELISA technique for treated groups including control (group I), pre treated with free albumin nanoparticles (group II), pre treated with GTP loaded albumin nanoparticles (group III), post treated with free albumin nanoparticles (group IV) and post treated with GTP loaded albumin nanoparticles (group V). The cell lysate was prepared using Nonidet-P40 (NP 40) buffer (150 mM NaCl, 1% Triton X 100 and 50 mM Tris, pH 8.0). Briefly, ELISA microtitre plate was coated with 100 µl primary antibody at a concentration of 1 µg/ml in carbonate/bicarbonate buffer (pH 9.6). The plate was covered with adhesive
and stored at 4°C overnight. Coating solution was removed after incubation, and the plate was washed twice with washing solution (Tris phosphate saline buffer - PBST). About 200 µl of blocking buffer (0.5% BSA in PBS) was added to each of the coated wells and was incubated overnight at 4°C. The plate was then washed twice with washing solution and 100 µl of sample (ascetic cells’ extract) was added to each well and incubated for 90 min at 37°C. The samples were removed and the plate was washed twice with washing solution. About 100 µl of Horse radish peroxidise (HRP) conjugated secondary antibody, diluted at an optimal concentration (according to the manufacturer’s instruction) in blocking buffer was added to each of the wells and incubated at room temperature for 2 h. The plate was then washed four times with washing solution. About 100 µl of the substrate [tetra methylbenzidine (TMB)] was dispensed to each of the wells. After sufficient colour development (~30 min), stop solution (1N H_{2}SO_{4}) of about 50 µl was added and the absorbance was recorded at 450 nm in an ELISA plate reader.

3.14. STABILITY OF GTP LOADED ALBUMIN NANOPARTICLES
Stability studies were carried out for 6 months (5 mg/ml GTP loaded albumin nanoparticles) at different temperatures like 4°C, room temperature (RT), 37°C and 45°C. The samples were withdrawn at regular intervals of one week for 3 months and 1 month for the remaining 3 months and the size of the particles and the surface charge was evaluated. After a period of 6 months, AFM, HR SEM, FTIR and XRD analysis were carried out, followed by in vitro drug release in simulated conditions (fasted and fed state) and release in the presence of alcohol as described in previous sections, to evaluate the cumulative percentage of drug release after a period of 6 months.

3.15. STATISTICAL ANALYSIS
All data were subjected to analysis of variance (ANOVA) and presented as mean ± S.D. The means were separated by Duncan’s multiple range test (DMRT) at \( p \leq 0.05 \).