Development and Evaluation of Lyophilized Stealth liposomal Phyllanthin: Pharmacokinetics and Toxicological studies

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Abstract

Though phyllanthin has several medical applications, especially in cancer treatment, oral administration of the drug is hampered by poor water solubility ultimately leading to inadequate bioavailability. To overcome the challenges related to solubility and increase oral bioavailability, current research focuses to develop phyllanthin-loaded liposomes by thin-film hydration and followed lyophilization. Several variants of conventional and pegylated liposomes were developed and their physicochemical properties were evaluated by various parameters such as size, zeta potential, and encapsulation efficiency. The optimal formulations (PHL2 & PHL7) were evaluated further for safety and efficacy. The compatibility of phyllanthin with excipients in selected formulations has been established by FTIR, DSC-TGA, and X-Ray diffraction studies. Toxicity and pharmacokinetic studies were conducted on rats to establish the safety and efficacy of the selected liposomal formulations. A sustained drug release pattern and enhanced bioavailability have been achieved with the liposomal formulations. The results from the current study indicate that phyllanthin-loaded pegylated liposomes are safest and ensure the maximum circulation half-life ($t_{1/2}$), MRT, and low elimination constant(K_{el}) compared to conventional liposomes and pure phyllanthin drugs. Thus pegylated liposomes can be used as a potential tool for oral administration of phyllanthin for chemotherapy.

1. Introduction

Phyllanthus amarus (*P. amarus*) is a broad-spectrum medicinal plant used in herbal medicine to treat a variety of diseases[1–3]. Several studies have reported antitumor activity associated with extract of *P.amarus* and recent studies attribute anticancer activity of *P.amarus* to the presence of bioactive lignans such as *Phyllanthin* and *Hypophyllanthin*[4–7]. *In-vivo* studies have shown that phyllanthin is a potent inhibitor of cytochrome p450 enzymes (Phase I enzymes) and exhibited cytotoxic effects on K-562 cells [4, 5].

The oral route is the most common form of drug administration and phyllanthin being poorly soluble in water (lipophilic) is highly unsuitable for oral administration. To surpass the solubility issue and improve the stability of phyllanthin in Gastrointestinal fluids, it can be formulated as liposomes. Liposomes are heterogeneous vesicular systems and consist of an aqueous core surrounded by one or more lipid bilayers[8–10]. Due to their morphology and physicochemical properties, they offer a wide array of advantages such as biocompatibility, biodegradability, enhanced efficacy, and therapeutic index, improved stability, improved and controlled pharmacokinetic and pharmacodynamics profiles of the drug, non-immunogenic, reduced toxicity, and active targeting[11–13]. Incredible progress has been made during the past few decades unraveling the potential of liposomes for drug delivery in the pharmaceutical and cosmetic industry. Liposomes are widely used for drug delivery in cancer therapy due to their unique ability to entrap both hydrophilic and lipophilic drugs and lead to a controlled release effect.

Although conventional liposomes offer several advantages and have morphology as cellular membranes, they are still foreign to the recipient immune system and cannot escape from phagocytosis[9, 14–16]. So,

the physicochemical properties of conventional liposomes are modulated for a specific purpose and one such modulation is to coat liposomes with a polymer such as polyethylene glycol (PEG), or polyvinyl alcohol (PVA). These are referred to as “Stealth liposomes” and those coated with PEG are referred to as “PEGylated liposomes”. The PEG coating extends the half-life of liposomes in the bloodstream by hindering the opsonization, which makes liposomes least susceptible to phagocytosis by the reticuloendothelial system (RES) and mononuclear phagocyte system (MPS)[14, 15]. Moreover, PEG coating increases the hydrodynamic size of the liposomes which further reduces renal clearance.

Advantages offered by pegylated liposomes lead current research to investigate the application of the pegylated liposomal formulation to deliver Phyllanthus via the oral route. One of the primary objectives of this research is to prepare and evaluate various liposomal formulations to identify optimal liposomal formulations. Additionally, the current research evaluates the *in-vitro* stability, toxicity, and pharmacokinetic performance of the optimal formulations in rats.

2. Materials

Phyllanthin was extracted and purified in-house where this research was carried out. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), and n-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (DSPE-MPEG-2000) were procured from Lipoid, Germany. Cholesterol, Pepsin, Pancreatin, Sodium taurocholate, Mannitol, Tween 20, and Stearic acid were all purchased from Sigma Aldrich, Germany. HPLC grade methanol and ethanol were procured from Merck, Chennai, India. Potassium chloride (KCl), Sodium hydroxide (NaOH) pellets, Potassium dihydrogen phosphate (KH_2PO_4), Calcium Chloride ($CaCl_2$), Sodium Chloride (NaCl), 0.1 N Hydrochloric acid (HCl) were procured from Reachem labs, Chennai, India.

3. Experimental

3.1. HPLC Assay

A validated high-performance liquid chromatography (HPLC) method was used to quantify the phyllanthin in the samples from *in vitro* and *in vivo* studies [4, 6, 17]. A quaternary gradient HPLC (Waters Delta Prep HPLC system, USA) system equipped with a Photo Diode Array (PDA) Detector (Waters 2998 PDA, USA) and a manual injector (Rheodyne, Cotati, CA, USA) with a 100 µL sample loop was used. HPLC method conditions were shown in Table 1.

Table 1
HPLC parameters for phyllanthin assay

Parameter	Required Setting
Elution mode	Isocratic
Mobile phase	Water to Methanol in the ratio of 34:66; (Filtered and Degassed)
Sample diluent	Mobile phase
Column	Waters Symmetry C18 250 mm × 4.6 mm, 5 µm
Flow rate	1.0 mL/min
Injection Volume	20 µL
Column temperature	30°C
Detection wavelength (λ max)	225 nm
Run time	40 minutes

[Table 1: **About Here]**

3.1.1. Preparation of Standard Solutions

The stock solution of phyllanthin was prepared by transferring 100 mg phyllanthin in a 100 mL volumetric flask containing 80 mL methanol, sonicated for 10 minutes and the volume was made up with methanol. A minimum of twelve standard solutions with concentrations ranging from 0.25 to 50 µg/mL were prepared by diluting the stock solution appropriately with the mobile phase. Phyllanthin spiked plasma samples were prepared by mixing 1 mL blank plasma with appropriate volumes of the standard phyllanthin solutions (100 µL) on the day of analysis. A blank was also prepared to contain 1 mL blank plasma. After preparation, the samples were stored at -20°C till the time of analysis. The phyllanthin concentration in samples was determined by the HPLC-UV method on the same day to avoid any degradation.

3.1.2 Preparation of Samples

The blood samples (0.3–0.4 mL) were withdrawn from the retro-orbital plexus and samples were collected into microcentrifuge tubes containing K2-EDTA (CML Biotech (P) Ltd, India). Then the samples were centrifuged at 4000 rpm for 5 min at 15˚C using a cold centrifuge (Remi Equipment Pvt. Limited, India). To 125 µL of plasma, 25 µL of internal standard (carbamazepine stock solution 10 µg mL^{-1} in methanol) was added and then vortexed (vortex mixer, Genei, Mumbai) for 60 seconds. The internal standard was added to ensure no analyte loss while sample preparation and the method produces a

consistent response. Then 500µL of methanol was added to precipitate proteins and vortexed for 5 min and centrifuged at 5000 rpm for 10 min. The supernatant was taken and dried in a vacuum oven at 40˚C. Dried samples were then redispersed in 100µL methanol and vortexed. The supernatant was separated and analyzed for drug content by the validated HPLC method.

1.2. Preparation of Conventional and Pegylated Liposomes

[Table 2: **About Here**]

Table 2
Composition of different formulations (conventional and pegylated liposomes) tested in this study

Formulation	Phyllanthin (mg)	DSPC (mg)	Cholesterol (mg)	DSPE MPEG2000 (mg)	Stearic Acid (mg)	Ratio of Phyllanthin: DSPC: Cholesterol: DSPEMPEG2000: Stearic Acid
PHL1	2.5	60	40	0	10	1:24:12:0:4
PHL2	5	60	40	0	10	1:12:8:0:2
PHL3	10	60	40	0	10	1:6:4:0:1
PHL4	5	70	30	0	10	1:14:6:0:2
PHL5	10	70	30	0	10	1:7:3:0:1
PHL6	5	60	40	5	10	1:12:8:1:2
PHL7	5	60	40	10	10	1:12:8:2:2
PHL8	5	60	40	15	10	1:12:8:3:2

Conventional and Pegylated liposomes containing phyllanthin were prepared using DSPC, Cholesterol and DSPE-MPEG2000 by film hydration technique. Stearic acid was added to impart charge on the surface of the vesicles and its concentration was kept constant in all the formulations. Different ratios of phyllanthin, DSPC, Cholesterol (CHOL), stearic acid and DSPE-MPEG2000 were mixed according to Table 2. Each composite was dissolved in chloroform/methanol (9:1 v/v) in a round-bottomed flask. The flask was then connected to a rotary evaporator (Buchi Rotavapor, Switzerland) and was rotated at a rate of 40 rpm at 65°C for 25 minutes before starting the vacuum pump.

Then Chloroform/methanol mixture was completely evaporated by applying a very low vacuum which left a thin lipid film on the walls of the flask. The lipid film was then hydrated at 65ºC (approximately 10ºC above the phase transition temperature (Tg) of DSPC) with 10 mL of phosphate-buffered saline

(PBS; pH-7.4) and vortexed (Vortex mixer, Genei Mumbai) for one hour. This resulted in large unilamellar vesicles (LUVs) being extruded 10 times through an extruder having 200 nm polycarbonate filter papers (Whatman, Denmark) at 65ºC above Tg of DSPC to obtain small unilamellar vesicles (SUVs). The extruded SUV suspension was then freeze-dried to obtain a fine powder of liposomes.

3.2.1 Lyophilization Cycle

The 5 mL of each extruded liposomal suspension was separately filled in 15 mL glass vials (Borosil, India) and subjected to freeze-drying using 5% (w/v) of mannitol as Cryoprotectant. The Freeze dryer (Lyodel, India) was pre-cooled to -40°C. 0.5 mL mannitol solution was added to all vials with liposomal formulation and the temperature was decreased at a rate of 0.5°C/min to -70°C and held at this temperature for 30 min. Then chamber pressure was decreased to 0.5 Torr and temperature was maintained at -16°C for 10 hrs. During secondary drying, the temperature was increased in a stepwise manner up to -16° C and to a final temperature of + 20°C, holding the temperature for 2 hrs at each step. Finally, the vials were screw-crimped immediately after withdrawing from the freeze dryer [15, 16, 18].

3.2.2. Reconstitution of lyophilized liposomes

The lyophilized liposomes were reconstituted in phosphate-buffered saline (PBS; pH-7.4) by gentle shaking for 30 seconds. After reconstitution, the freeze-dried formulations were characterized for the appearance of the cake, vesicle size, size distribution (Polydispersity index-PDI), Encapsulation Efficiency (EE), and *In-vitro* drug release.

3.3. Evaluation of Liposomes

Several parameters were measured to evaluate the formulated liposomes such as Encapsulation Efficiency (%EE), Particle Size, Zeta Potential, Poly Dispersity Index (PDI), X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy, Transmission Electron Microscopy (TEM), Differential Scanning Calorimetry and Thermogravimetric analysis (DSC-TGA). *In-Vitro* drug release, toxicity, and stability studies were also performed. All animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare.

3.3.1 Encapsulation Efficiency (% EE) Determination

The % EE of liposomes was determined by using the ultracentrifugation technique. 2mL of liposomes suspension was centrifuged at 25,000 rpm for two hours at a controlled temperature of 4°C (Remi cooling centrifuge). The supernatant containing free drug was withdrawn and estimated by the HPLC method described in section 3.1. The % EE was estimated using Eq. 1. This procedure was repeated three times per formulation and average of three replicates was reported.

$$\text{Encapsulation Efficiency } (\%\ \text{EE}) = \frac{\text{Amount of drug in supernatant}}{\text{Amount of drug initially taken for liposome preparation}} \times 100 \quad \text{Eq: 1}$$

3.3.2 Size, Size Distribution, polydispersity index, and Zeta Potential Analysis

The liposomal suspension was diluted 10 times with 0.1 M sodium chloride solution. The size and size distribution of liposomes was determined using DLS (dynamic light scattering) (Nano partica, Horiba Scientific, Japan), by taking the average of 3 measurements, whereas zeta potential was estimated based on electrophoretic mobility under an electric field, as an average of 3 measurements. The polydispersity index (PDI) which is a dimensionless number indicating the width of the size distribution was also measured.

3.3.3 Shape and Surface Morphology by TEM

The particle size, shape, and surface layers of the optimal liposomal formulations were examined by transmission electron microscopy. 20µL of the sample was deposited over carbon-coated copper grids with 200 mesh for 60 s and dried. Excess dispersion was blotted from the grid with filter paper to form a thin-film specimen. Then they were stained with 2%uranyl acetate, air-dried, and examined under a transmission electron microscope (Hitachi H-7500, Japan).

3.3.4 FTIR Analysis

FT-IR spectra were recorded on a Bruker Spectrophotometer, Switzerland. Phyllanthin, a pure drug, and its liposomal formulations were prepared in KBr discs prepared at a hydrostatic pressure of 5 tones cm^{-2} for 2 mins. The scanning range was 400–4000 cm^{-1} with a resolution of 1 cm^{-1}. The FT-IR spectra of pure phyllanthin, DSPC, cholesterol, DSPEMPEG2000, stearic acid, mannitol, and their corresponding selected lyophilized liposomal formulation were compared.

3.3.5 DSC-TGA Analysis

DSC-TGA analysis was performed to study the thermal stability and changes in crystallinity over a range of temperatures. Thermographs of pure drug, blank formulations, and selected lyophilized liposomal formulations were recorded using SDT Q600 V20.9 Build 20, TA Instruments, USA. A known mass of sample (4–6 mg) was transferred to an aluminum pan and crimped sealed with a lid. An empty aluminum pan sealed with a lid was used as a reference sample. Samples were scanned from 25°C to 400°C at a rate of 10°C min^{-1} under an N_2 purge.

3.3.6 XRD Analysis

The powder X-ray diffraction pattern of phyllanthin and selected liposomes in lyophilized powder forms was recorded using an X-RD instrument (PANalytical XPERT-PRO, Netherlands). The selected samples were analyzed in the 2Θ angle range of 5 to 89.99.

3.4. *In vitro* Dissolution Studies

The *in vitro* drug release for pure drug and liposomal formulations was performed using the dialysis membrane method. Before use, the dialysis membrane (Himedia, molecular weight cut off 12,000) was activated by washing the membrane under running water for 6 hrs to remove glycerol. Subsequently, sulfur was removed by treatment with 0.3% sodium sulfide for 1 min, followed by acidification (0.2% sulfuric acid) of the membrane. Finally, it was washed with water repeatedly to remove the excess acid. The *in-vitro* diffusion studies were carried out in two 500 mL beakers one containing 250 mL of 0.1N HCl (pH 1.2) and the other with 250mL of phosphate buffer (pH 7.4). Both the buffer systems contain 0.1% Tween 80 to solubilize the released drug and were equilibrated to 37 ± 0.5 ºC. The dialysis membrane was filled with test products, and both ends of the membrane were sealed. The dialysis membrane containing liposomes was suspended in the release medium (0.1 N HCl for 2 hrs followed by pH 7.4 phosphate buffer for 22 hrs) which was under slow constant stirring. The concentration of phyllanthin in the release medium was determined by withdrawing 2 mL of the sample at regular intervals for 24 hours and immediately replaced with a respective medium by the same quantity. The samples were analyzed using the HPLC method (section 3.1) and % cumulative drug release was calculated. All the drug release studies were conducted in triplicate.

3.5. Stability of Liposomal Formulations

The stability of selected freeze-dried conventional and pegylated liposomal formulations containing phyllanthin in different simulated Gastrointestinal fluids (Simulated Gastric Fluid (SGF), pH 1.2, and Simulated Intestinal Fluid (SIF), pH 6.8) was evaluated. The simulated gastric fluid (SGF) contains 0.2% NaCl, pepsin, 0.7%, and HCl with a pH of 1.2. Simulated intestinal fluid (SIF) consists of 0.685 M monobasic potassium phosphate, 1% NaOH, and 1% pancreatin with pH 6.8. To simulate the effect of bile salts, 3 mM sodium taurocholate was added to the SIF.

The Phyllanthin conventional liposomes (PHL2) and pegylated liposomes (PHL7) were reconstituted with the required volume of PBS (pH 7.4) before use. 1mL of each reconstituted formulation was added to 9 mL of each simulated GI fluid. The samples were incubated in a water bath at 37 ± 1°C and shaken at 100 rpm for 2 hrs and 6 hrs in SGF and SIF, respectively. The enzyme activity in the samples was completely inhibited by the addition of an equal volume of 0.1 M ice-cold sodium hydroxide to SGF and 0.1 M HCl to SIF. The samples were then evaluated for vesicle size, PDI, zeta potential, and % encapsulation efficiency as described in section 3.3 [18].

3.6. Toxicity Studies

Swiss mice (female) weighing 20-25g (Mahaveer enterprises, Hyderabad, India) were randomly divided into three groups (6 mice per group). All formulations were administered orally at a dose of 1000 mg of phyllanthin/kg body weight. Group I served as the control group and was administered drug-free pegylated liposomes, Group II and III were administered phyllanthin-loaded conventional liposomes (PHL2) and phyllanthin-loaded pegylated liposomes (PHL7) respectively. After 14 days of the administration, blood samples from the retro-orbital plexus were collected in K_2-EDTA microcentrifuge tubes and centrifuged at 3000 rpm for 5 min to separate plasma. The plasma was then analyzed for the

levels of various markers. Blood urea nitrogen (BUN), plasma urea, and plasma creatinine levels were determined for evaluating nephrotoxicity while plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels were determined for evaluating hepatotoxicity. All the biochemical parameters were determined by using an autoanalyzer (RM4000 Biochemical systems International, Italy) according to the manufacturer's guidelines for the diagnostic kits obtained from Span diagnostics (Span diagnostics LTD., India).

3.7. Pharmacokinetic Evaluation of Phyllanthin Liposomes

Healthy Sprague Dawley rats of either sex weighing about 220–250 g were procured from Mahaveer enterprises, Hyderabad. The animals were acclimatized under standard environment conditions (23 ± 2ºC, 55 ± 5% relative humidity, 12 hrs light/ dark cycle) for one week. The pure phyllanthin and selected liposomal formulations (PHL2 & PHL7) were administered orally at a dose equivalent to 5 mg/kg body weight. The freeze-dried formulations were suitably reconstituted with saline to achieve the desired drug dose in 0.5mL volume, which was administered to animals and the pure drug was suspended in the suspension of Sodium CMC and administered to animals. Group I was administered with pure phyllanthin whereas groups II & III were administered reconstituted phyllanthin conventional liposomes (PHL2) and pegylated liposomes containing phyllanthin (PHL7) respectively. The blood samples (0.3 mL) were withdrawn from the retro-orbital plexus at 0, 0.5, 1, 2, 4, 8, 12, 24, and 36 hours after oral administration. The blood samples were prepared as described in section 3.1.2 and analyzed for drug content by the validated HPLC method. The plasma concentration over time was analyzed by non-compartmental extravascular method, using Kinetica software (Thermo scientific USA). The mean pharmacokinetic parameters ($AUC_{0\rightarrow\infty}$, $AUMC_{0\rightarrow\infty}$, C_{max}, T_{max}, $t_{1/2}$, K_{el}, MRT, Cl, and V_z) were calculated from plasma drug concentration ($ng{\cdot}mL^{-1}$) - time (hrs) plots using Kinetica software by applying a log-linear trapezoidal rule. This study is reported in accordance with ARRIVE guidelines. The experiment was performed in accordance with relevant guidelines and regulations.

4. Results And Discussion

4.1. Evaluation of Physicochemical Properties of Liposomes

The phyllanthin-loaded liposomes were successfully prepared by film hydration technique. The results of physicochemical properties of prepared liposomes were shown in Table 3. The percentage drug content was in the range of 99.12 ± 1.32% to 99.89 ± 1.10% and was found to be identical irrespective of liposomal formulations. These results indicate that there was no loss of the drug during the formulation of liposomes.

Table 3
Physicochemical properties of conventional and pegylated liposomes

Formulation	Assay (%)	Size(nm)	%EE	PDI	Zeta Potential(mV)
PHL1	99.12 ± 1.32	130 ± 2.03	86.47 ± 0.19	0.202 ± 0.012	-32.37 ± 1.10
PHL2	99.19 ± 1.22	133 ± 5.21	82.61 ± 0.16	0.254 ± 0.006	-32.97 ± 1.92
PHL3	99.89 ± 1.10	134 ± 3.06	66.43 ± 0.20	0.311 ± 0.007	-33.4 ± 0.45
PHL4	99.68 ± 1.23	186 ± 4.48	78.36 ± 0.20	0.234 ± 0.016	-37.25 ± 2.44
PHL5	99.58 ± 0.89	198 ± 4.33	74.49 ± 0.30	0.299 ± 0.006	-36.05 ± 0.89
PHL6	99.87 ± 1.20	129 ± 2.73	83.68 ± 0.54	0.138 ± 0.014	-33.88 ± 0.93
PHL7	99.82 ± 1.24	132 ± 2.65	82.14 ± 0.55	0.211 ± 0.006	-34.22 ± 2.20
PHL8	99.78 ± 1.22	136 ± 2.18	82.51 ± 0.25	0.274 ± 0.007	-34.82 ± 1.74

[Table 3: **About Here**]

The mean vesicle size of phyllanthin liposomes was found to be in the range of 130 ± 2.03 nm to 198 ± 4.33 nm. The polydispersity index (PDI) was in the range of 0.126 ± 0.77 to 0.372 ± 0.2. These results indicate that the liposomal dispersion approached a monodisperse stable system and even delivers the drug effectively, owing to the large surface area of its nanosized vesicles. The zeta potential values of liposomes were found to be in the range of -32.37 ± 1.10 mV to -37.25 ± 2.44 mV as listed in Table 3. The obtained zeta potential values indicate the presence of sufficient charge to inhibit aggregation of vesicles due to electric repulsion. Excellent %EE has been achieved for various liposomal formulations as listed in Table 3 which could be due to sufficient fatty acid chain length of selected DPSC.

4.1. Stability in simulated GI fluids

In vitro stability studies in simulated gastrointestinal fluids revealed that conventional liposomes (PHL2 & HPL2) were unstable in both SGF and SIF as presented in Table 4. The excess hydrogen ions, present in the external environment can diffuse to the inner aqueous phase of liposomes, made up of phospholipids, and destabilize them. Whereas the bile salt monomers can also permeate into lipid bilayers and may disrupt the vesicular structures. Furthermore, the cholic and taurocholic acid in the gastrointestinal tract can also increase the permeability of liposomes, whereas the pancreatic lipases tend to lyse the liposomes [19].

Table 4
Comparison of various parameters of selected liposomes before and after subjecting to different simulated GI fluids

		Parameters					
		Vesicle size (nm)		Zeta potential		% Encapsulation	
Formulation	Medium	Initial	Final	Initial	Final	Initial	Final
PHL2	SGF pH 1.2	140 ± 3.48	195 ± 4.11	-33.22 ± 1.6	-30.52 ± 2.85	81.17 ± 0.14	69.25 ± 0.25
	SIF pH 6.8	140 ± 3.48	162 ± 3.52	-33.22 ± 1.6	-31.34 ± 2.85	81.17 ± 0.14	74.34 ± 0.25
PHL7	SGF pH 1.2	137 ± 3.78	166 ± 2.86	-33.85 ± 1.52	-30.52 ± 2.85	79.29 ± 0.11	73.84 ± 0.18
	SIF pH 6.8	137 ± 3.78	144 ± 3.12	-33.85 ± 1.52	-30.22 ± 2.64	79.29 ± 0.11	77.45 ± 0.18

[Table 4: **About Here**]

These rupturing and lysing of the liposomes can be accounted for the changes in particle size and encapsulation efficiency, being indicative of the destabilization of the system. Poor stability of conventional liposomes could also be attributed to the aggregation owing to the presence of the opposite charge on the surface of liposomes compared to that of the exterior environment.

On the other hand, pegylated liposomes (PHL7) were found to be stable in all mediums in terms of particle size, zeta potential, and encapsulation efficiency. The reason behind their stability could be the protective role of PEGylation, which prevents the exposure of the phospholipids to the harsh conditions of the gastrointestinal tract, suggestive of the robustness of formulation [18]. Even previous studies [20, 21]reported that pegylation resists the rupturing of liposomes by preventing the action of bile salts like sodium taurocholate and other gastric enzymes.

4.2. *In*-vitro Dissolution by Dialysis

In-vitro drug release profiles of tested products were shown in Fig. 1. Over 24 hrs, only 34.52% of the pure drug was released. All the liposomal formulations, irrespective of chemical composition, released between 5% and 10% of the drug in the acid medium (pH 1.2) for initial two hrs. When the release medium was changed to phosphate buffer (pH 7.4), a sudden spike in drug release rate was observed for all liposomal formulations. The drug release was found to be in the range of 59.84–67.65% for conventional formulations whereas pegylated formulations have shown 71.65–84.85% of drug release over 24 hrs. As anticipated both conventional and pegylated liposomes have shown slow and extended-release when compared to pure drug. The extended-release profiles of liposomal formulations can be

attributed to increased stability due to the incorporation of cholesterol into liposomes. Additionally, pegylated liposomes have a better rate of release and shown a higher % drug release when compared to conventional liposomes as evident from Fig. 1.

[Figure 1: **About Here]**

As the ratio of DSPEMPEG2000 to lipid concentration increased (from PHL6 to PHL7), the rate of drug release from the vesicles increased, but a further increase of DSPE-MPEG2000 concentration (15%) in PHL8 does not show any significant increase in the rate of drug release. An interesting release trend was observed for conventional liposomes, PHL3 and PHL5, where 60.27% of the drug was released at the 5th hour for PHL3 and 62.49% of the drug released at the 4th hour for PHL5. These formulations contain relatively higher drug content (10 mg) which might be a factor in decreased stability of liposomes leading to precipitation followed by burst release. Moreover, PHL5 was relatively less stable than PHL3 due to higher lipid concentration.

The drug release data were analyzed as per zero-order, first-order, Higuchi, Hixson-Crowell (erosion), and Peppa's equation models to assess the release kinetics and mechanism of drug release from the liposomes prepared. The drug release of all liposomal formulations has a better fit to first-order kinetics and the mechanism of drug release appears to be diffusion-based as indicated by high correlation coefficients for Higuchi's equation. The 'n' values in the Peppas equation strongly suggest a non-Fickian diffusion mechanism for drug release mechanism from the phyllanthin-loaded liposome formulations. The results of the data analysis of drug release profiles were summarized in Table 5.

Table 5
Correlation coefficient (r) values in the analysis of release data of conventional and pegylated liposomes containing phyllanthin (PHL1-PHL8)

	Correlation Coefficient (r)							
Formulation	Zero-order	First-order	Higuchi	Peppas	Erosion	Ko ($mg \cdot hr^{-1}$)	K_1 (hr^{-1})	'n' in Peppas equation
PHL1	0.9476	0.9823	0.9849	0.9695	0.9731	2.676	0.0392	0.606
PHL2	0.9434	0.9829	0.9849	0.9726	0.9721	2.867	0.0438	0.694
PHL3	0.7688	0.7746	0.8854	0.9252	0.788	3.048	0.0484	0.807
PHL4	0.9257	0.967	0.9803	0.9586	0.9555	2.515	0.0368	0.659
PHL5	0.7036	0.6907	0.8408	0.9033	0.7162	2.888	0.0438	0.883
PHL6	0.9033	0.956	0.9675	0.9571	0.9423	3.246	0.0553	0.702
PHL7	0.9011	0.9716	0.9664	0.9618	0.9534	3.879	0.0852	0.776
PHL8	0.8933	0.967	0.9633	0.9581	0.9482	3.881	0.0852	0.796

[Table 5:**About Here]**

The formulations PHL2 (conventional) and PHL7 (pegylated) showed optimum particle size, better entrapment efficiency, and maximum drug release in a controlled manner when compared to the other liposomal formulations. Hence these two were selected for further studies such as *in vivo* drug release studies, stability studies, XRD, DSC-TGA, and FT-IR analysis.

4.3. Drug and Excipient Interactions

A significant deviation in pharmacokinetic and pharmacodynamic properties will arise if there is a physical or chemical interaction of the drug with excipients. TEM, FT-IR, DSC-TGA, and X-ray diffraction analyses have been conducted to identify the interactions between drug and excipients in selected liposomal formulations (PHL2 and PHL7).

4.3.1 TEM Analysis

Figure 2 shows the photomicrographs of liposomes of PHL2 and PHL7 which conform the unilamellar structure of liposomes. The obtained size was in good correlation with that from the particle size analyzer. Additionally, the spherical structure of these formulations was also demonstrated.

[Figure 2: **About Here]**

4.3.2 FTIR Analysis

Phyllanthin has characteristic FT-IR absorption bands at 2917.93 cm^{-1}, 2959.97 cm^{-1}, 2998.9 cm^{-1} (C-H stretching (aromatic), 2850.37cm^{-1}(C-H stretching (aliphatic), 1041.73 cm^{-1}, 1108 cm^{-1}, 1140.61 cm^{-1}, 1159.18 cm^{-1}, 1179.69 cm^{-1}(C-O-C stretching) as shown in Fig. 3.

[Figure 3: **About Here]**

The FTIR spectrum of PHL2 have characteristic absorption band at 2850.33 cm^{-1}, 2917.85 cm^{-1}, 2983.94 cm^{-1} due to CH stretching, and 1081.41 cm^{-1}, 1140.41 cm^{-1}, 1159.67 cm^{-1} bands due to C-O-C stretching. The PHL7 has characteristic absorption bands at 2850.58 cm^{-1}, 2917.88 cm^{-1} due to CH stretching, and 1084.99 cm^{-1} due to C-O-C stretching. In case of PHL2, the characteristic CH stretching band shifted to 2850.33 cm^{-1}, 2917.85 cm^{-1}, 2983.94 cm^{-1} and C-O-C to 1081.41 cm^{-1}, 1140.41 cm^{-1}, 1159.67 cm^{-1} respectively with reduced in intensity. In the case of phyllanthin-loaded pegylated liposomes-PHL7, the characteristic CH stretching band shifted to 2850.58 cm^{-1}, 2917.88 $cm^{-1,}$ and C-O-C to 1084.99 cm^{-1} respectively. The peaks in IR spectra of the pure drug were present in IR spectra of the liposomes, without disappearance and any change in the position. It was concluded that phyllanthin is compatible with DSPC, Cholesterol, Stearic acid, and DSPC-MPEG2000 and is free from chemical or physical interaction.

4.3.3. XRD Analysis

The XRD patterns of phyllanthin, blank formulation of conventional liposomes, DSPE MPEG2000, selected conventional (PHL2) and pegylated liposomes (PHL7) loaded with phyllanthin were shown in Fig. 4.

[Figure 4: **About Here]**

For pure phyllanthin sharps peaks at diffraction angles (2Θ) 11°,14°,17°, 19°, 22°, and 24° with an intensity of more than 3000 were observed. This suggests phyllanthin is a crystalline material. The formulations of phyllanthin both conventional and pegylated liposomes showed similar 2Θ characteristics as of pure drug with a significant shift in 2Θ and reduction in peak intensities. It can be concluded that the phyllanthin is free from interaction with DSPC, cholesterol, stearic acid, and DSPE-MPEG2000 and is present in the amorphous form.

4.3.4 DSC-TGA Analysis

Phyllanthin (pure) has a small endothermic peak characteristic of melting at 97.7°C as shown in Fig. 5, while an endothermic peak at 298.25°C indicates the degradation of the drug. The blank conventional formulation and DSPE-MPEG2000 have a glass transition temperature (Tg) of 64.27°C and 58.6°C respectively. PHL2 has a Tg of 66.79°C, while PHL7 exhibited two endothermic peaks: one at 58.46°C characteristics of the DSPE-MPEG2000, and the other at 65.54°C which is characteristic of other

excipients. Both formulations (PHL2 and PHL7) exhibited a characteristic peak for phyllanthin at 97.7°C. Phyllanthin showed a high degree of thermal stability in PHL2 and PHL7, an additional benefit, which is evident from the lack of degradation peak around 298°C as seen in pure drug. As the melting points of the drug have not shown any significant differences in the thermograms of DSC-TGA it may be concluded that the drug has not formed any inclusive complex with excipients and is free from any physical or chemical interaction.

[Figure 5: **About Here]**

4.3.5 Toxicity studies

The toxicity profile of the test products was studied using different toxicity markers. Table 6 summarizes the results of ALT, AST, creatinine, plasma urea, and BUN in plasma samples collected from treated animal groups. There is no significant difference ($p > 0.05$) in the levels of toxic markers between groups treated with different liposomal formulations (PHL2 & PHL7) and the control group. From the previous reports on different anticancer drug formulations, the safer toxicity range for different hepato- and nephrotoxic parameters was found to be AST (13.68–19.26 IU/L), ALT (49.25–64.81 IU/L), BUN (6.54–11.25 IU/L), plasma urea (16.27–22.75 IU/L) and Creatinine (0.65–1.25 IU/L) [18]. The concentrations of all toxicity markers in the treated groups were within the safe toxic range and as summarized in below Table 6 and both formulations are relatively safe for *in vivo* use.

Table 6
Summary of biochemical toxicity study. Results are expressed as Mean ± sd (n = 6).

Group	AST ($IU{\cdot}L^{-1}$)	ALT ($IU{\cdot}L^{-1}$)	BUN ($IU{\cdot}L^{-1}$)	Plasma urea ($IU{\cdot}L^{-1}$)	Creatinine ($IU{\cdot}L^{-1}$)
I. Control	13.52 ± 2.17	48 ± 5.62	7.48 ± 1.96	16.4 ± 3.75	0.65 ± 0.14
II. PHL2	15.64 ± 1.98	50.26 ± 3.84	8.82 ± 2.67	18.65 ± 2.34	0.82 ± 0.11
III. PHL7	17.29 ± 3.22	52.7 ± 7.28	10.65 ± 4.72	17.48 ± 3.16	0.96 ± 0.16

[Table 6: **About Here]**

4.3.6 Pharmacokinetic Studies

The Plasma drug concentrations over a period for test formulations are shown in Figure 6 and associated pharmacokinetic parameters were shown in Table 7. C_{max} values for PHL2 and PHL7 were increased by 2.7 and 4.3-fold respectively, compared to pure drug demonstrating the enhanced oral bioavailability of phyllanthin. PHL7 has the highest T_{max} followed by PHL2 and finally, pure drug has the lowest. T_{max} values support the tendency of liposomal formulations to release the drug in a controlled manner compared to the pure phyllanthin drug. The $AUC_{0\rightarrow\infty}$ values for PHL2 and PHL7 were improved by 2.9 and 5.8-fold respectively, compared to pure drug. Similarly, a 3.9 and 9.1-fold increase in the $AUMC_{0-\infty}$ (ng·h

·mL^{-1}) values for PHL2 and PHL7 respectively, was observed when compared to pure drug. MRT and $t_{1/2}$ values were also improved significantly for liposomal formulations compared to the pure drug as shown in Table 6. The MRT and $t_{1/2}$ values of test products indicate that the systemic circulation time of phyllanthin was improved for the liposomal products compared to the pure drug, especially from Pegylated liposomes (PHL7). The values for clearance (Cl) and Volume of Distribution (Vz) were superior for liposomal formulations, especially pegylated form when compared to pure drug. The *in vivo* pharmacokinetic studies revealed that phyllanthin-loaded pegylated liposomes have superior pharmacokinetics and this form is desired and highly effective with the highest oral bioavailability (C_{max}), long circulation ($t_{1/2}$), better residence time (MRT), and low elimination rate (K_{el}) compared with pure phyllanthin drug.

Table 7
Pharmacokinetic Data of Test Products

	Test products		
Pharmacokinetic parameter	Pure Phyllanthin 5 $mg{\cdot}kg^{-1}$	Conventional liposomes (PHL2)	Pegylated liposomes (PHL7)
C_{max} ($ng{\cdot}mL^{-1}$)	530.94 ± 23.88	1417.85 ± 55.65	2279.86 ± 105.47
T_{max} (hrs)	1.17 ± 0.12	2.13 ± 0.09	4.07 ± 0.036
$AUC_{0-\infty}$(ng. $h{\cdot}mL^{-1}$)	5265.30 ± 275.52	15217.60 ± 987.96	30810.23 ± 2587.96
$AUMC_{0-\infty}$ (ng. $h{\cdot}mL^{-1}$)	36920.13 ± 1801.55	142783.33 ± 10755.96	334518.33 ± 28843.97
MRT (hrs)	7.03 ± 0.04	9.36 ± 0.11	10.85 ± 0.027
K_{el}^{-1} (hr)	-0.2 ± 0.01	-0.13 ± 0.02	-0.12 ± 0.004
$t_{1/2}$ (hrs)	3.41 ± 0.12	5.46 ± 0.004	5.91 ± 0.264
Cl ($mL\ h^{-1}$)	960.11 ± 50.29	334.25 ± 21.89	167.35 ± 15.04
V_z (mL)	4748.99 ± 407.04	2414.14 ± 101.05	1449.15 ± 197.82

[Table 7: **About Here]**

5. Conclusions

Phyllanthin-loaded pegylated liposomes were successfully developed by film hydration Technique. The aqueous solubility and poor oral bioavailability of phyllanthin has been addressed by liposomal technology. The obtained size of liposomes for selected formulations (PHL2 & PHL7) is suitable for better oral absorption. In addition, the TEM images conforms that developed liposomes are unilamelar nano-

sized spherical vesicles. The drug and excipients used in formulation were compatible with minimal to no interaction. All the toxicity indicators are with in safe limits for the liposomal formulations when administered orally. Liposomal formulations exhibited sustained release of drug as supported by pharmacokinetic data. Overall, the pegylated formulations has shown superior characteristics in terms of stability, physicochemical, and pharmacokinetic properties. The enhanced bioavailability of liposomal formulation definitely helpful for producing pharcological activity at relatively lower dose compared to free drug.

Declarations

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Availability of Data and Materials

The datasets generated and analyzed during this study are available from the corresponding author on request.

Ethics approval and consent to participate

All animal protocols were as approved by Institutional Ethical Committee (IAEC) of Regd. No. 516/01/A/CPCSEA, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Madhukiran Parvathaneni: Methodology, investigation, writing—original draft, visualization, conceptualization, supervision, funding acquisition.

Naga P. D. Boppana: Formal analysis,investigation, writing—review & editing. All authors read and approved the final manuscript.

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Figures

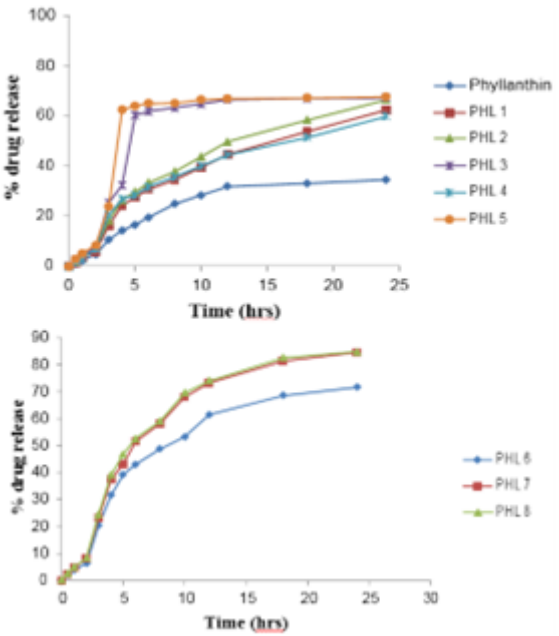


Figure 1

In-vitro drug release profiles of test products

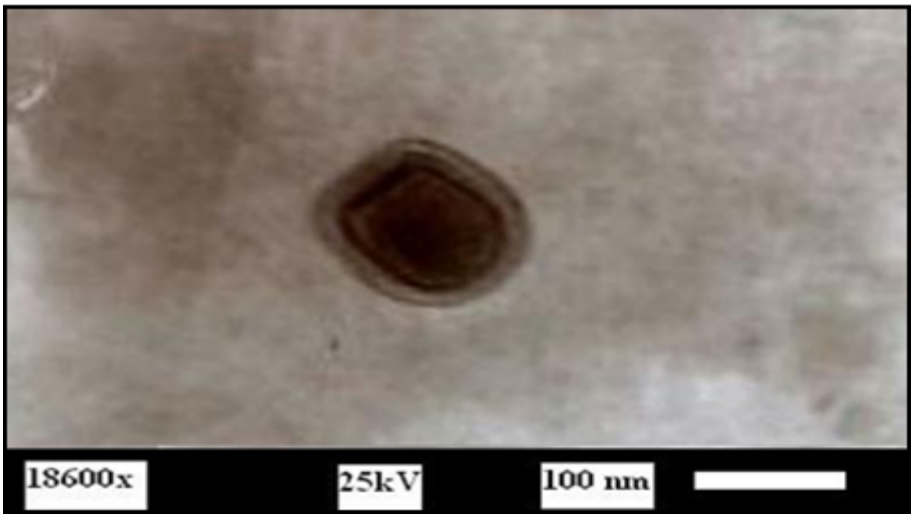


Figure 2

TEM Image of PHL7

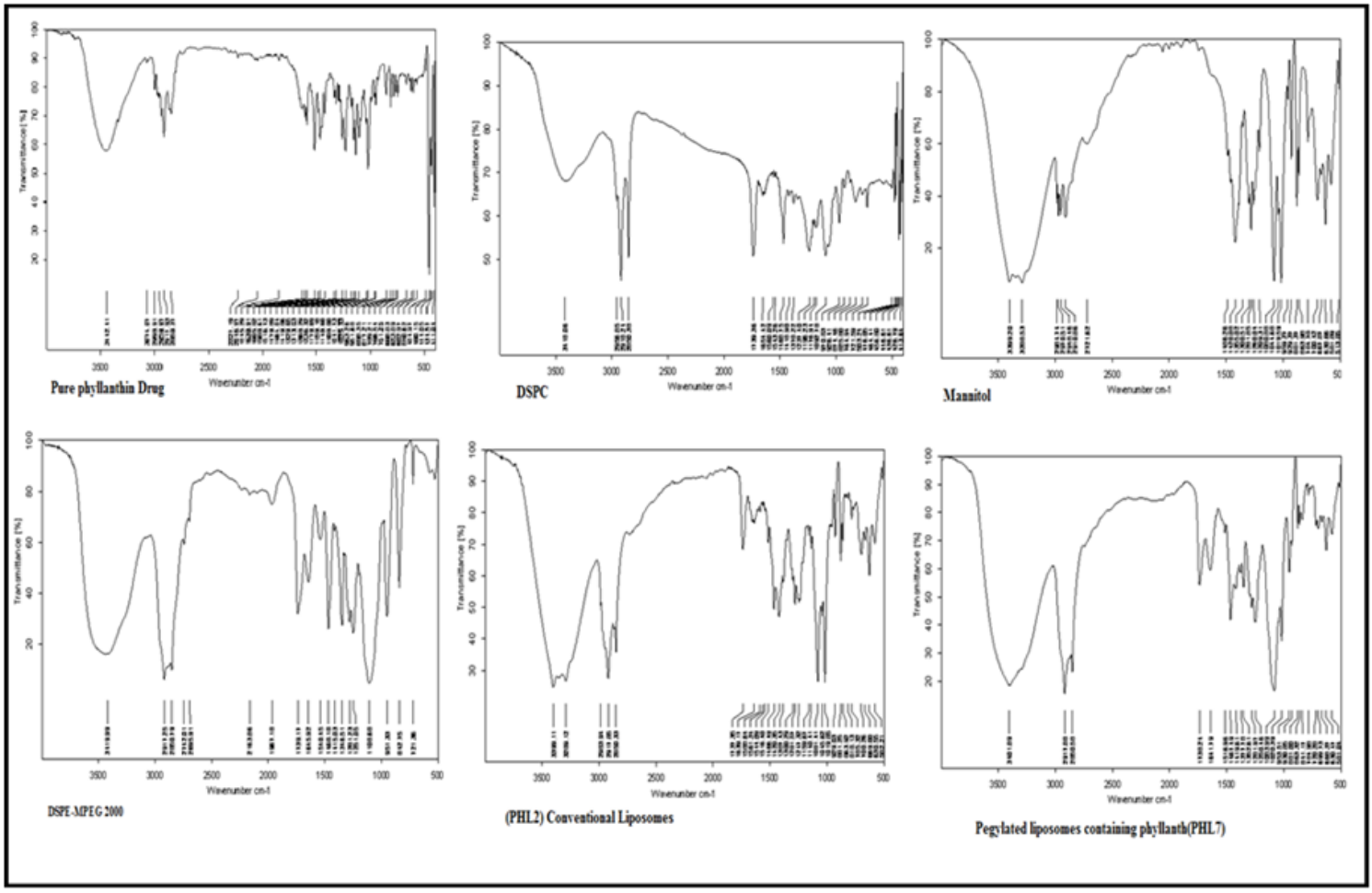


Figure 3

FTIR spectra of test products

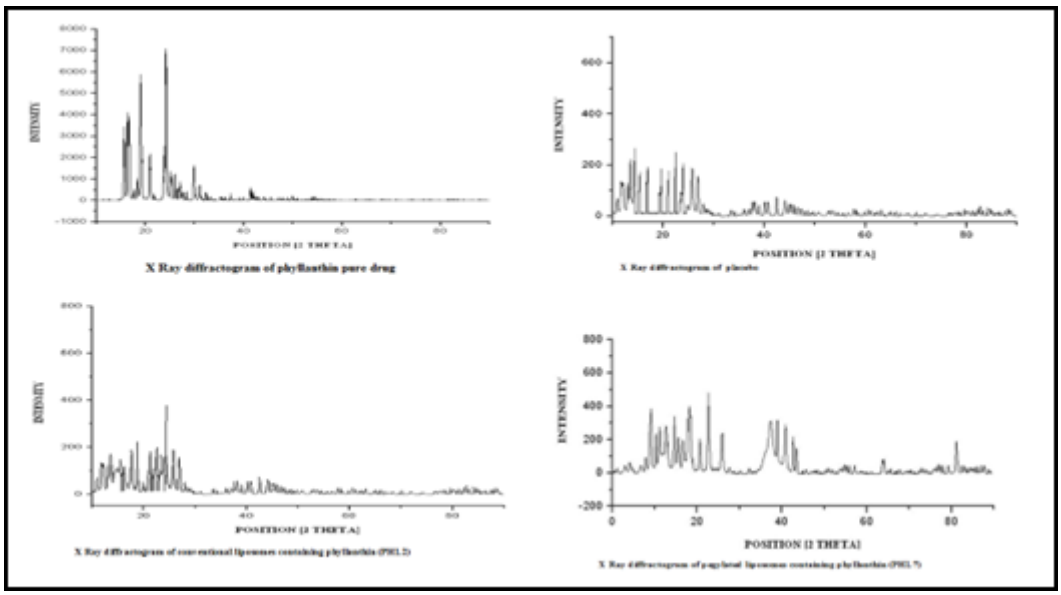
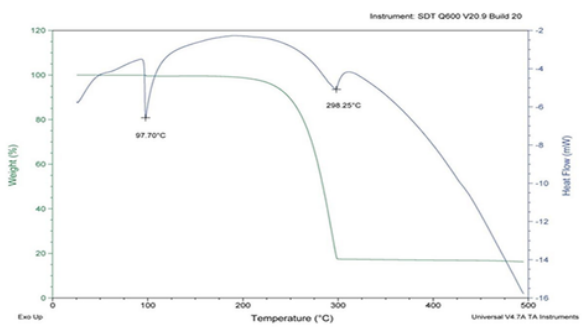
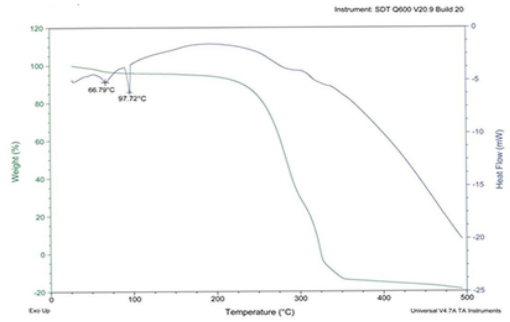


Figure 4

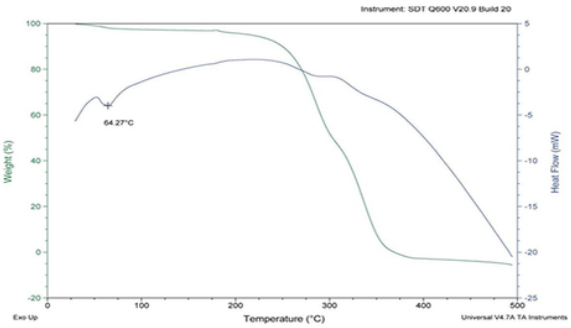
X-ray Diffractograms of test products



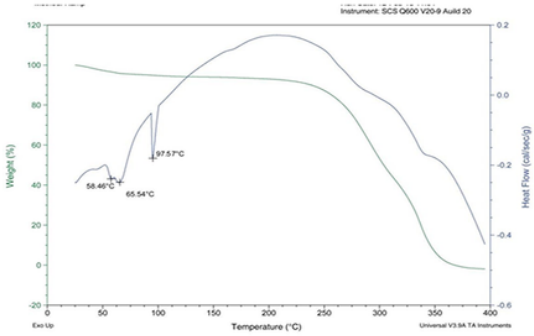
DSC-TGA thermogram of phyllanthin pure drug



DSC-TGA thermogram of conventional liposomes containing phyllanthin (PHL2)



DSC-TGA thermogram of dummy liposomes



DSC-TGA thermogram of pegylated liposomes containing phyllanthin (PHL7)

Figure 5

TGA-DSC Thermal curves of Test Products

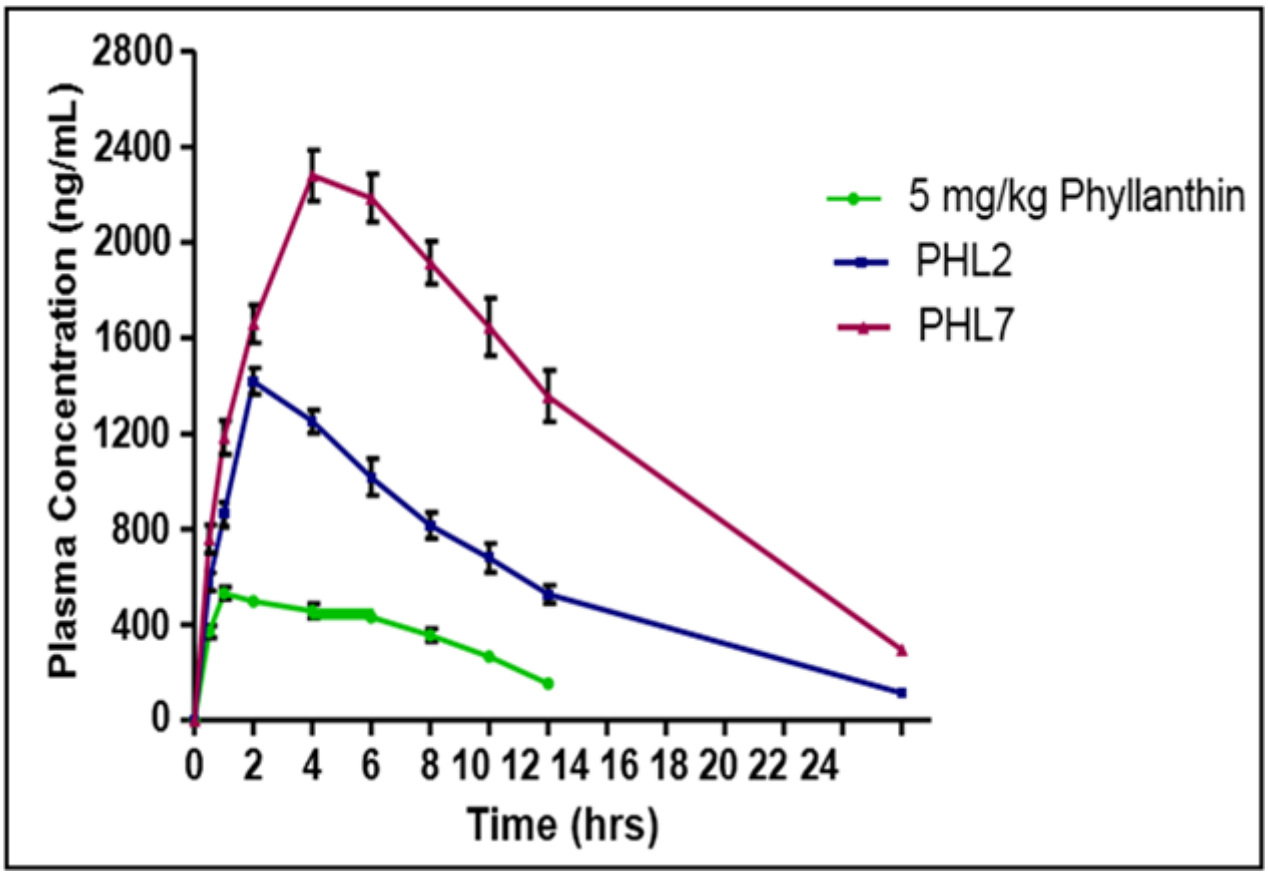


Figure 6

Plasma drug concentration over time for test products