Pharmacokinetic and Toxicological Evaluation of Hypophyllanthin Loaded Liposomes

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Abstract

Phyllanthus amarus (*P. amarus*) is a broad-spectrum medicinal plant and studies have reported anticancer activity of *P. amarus* to the presence of bioactive lignans such as Phyllanthin and Hypophyllanthin. *In vivo* studies have shown that hypophyllanthin is a potent inhibitor of cytochrome p450 enzymes (Phase I enzymes) and exhibits cytotoxic effects on K-562 cells. The most common route of drug administration is via oral, and hypophyllanthin, being poorly soluble in water (lipophilic), is highly unsuitable for oral administration. To improve the aqueous solubility and pharmacokinetic profile, hypophyllanthin is formulated as stealth liposomes. The liposomes are developed using film hydration technique and are evaluated for various physicochemical parameters. The synthesized liposomes were found to be in the size range of 128 to 205 nm, PDI was found in the range of 0.2 to 0.3, encapsulation efficiency was found to be in the range 62 to 85, the zeta potential was found in the range of -32 to -40 mV respectively. The pegylated liposome (HPL7) showed superior pharmacokinetics and 86.05% of drug was released over 24 hours. The safety and efficacy of test formulations were established by toxicity and pharmacokinetic studies. The obtained results indicates that, pegylated liposomes are safest and ensures the maximum circulation half life ($t_{1/2}$), MRT and low elemination constant(K_{el}) compared to conventional liposomes and pure phyllanthin drug.

1. Introduction

Liposomes as drug delivery agents is highly investigated and their applications in food, drug and cosmetic industry are endless[1–5]. Liposomes are heterogeneous, spherical vesicular systems with phospholipid bilayer enclosing aqueous compartment. Their unique ability to encapsulate both hydrophilic and lipophilic drugs aids in delivery of wide span of drugs[3, 4, 6]. Liposomal formulations have shown improved therapeutic index, which in turn reduces the drug toxicity and better patient compliance, higher oral absorption, and prolonged systemic blood circulation[2, 7, 8]. Liposomes are also extensively used in targeted delivery of drugs[9–12].

Liposomes have been extensively studied and developed for drug delivery. A variety of liposomes technologies have been developed to address specific challenges associated with the drug substance. Recently, liposomes found immense applications in drug delivery for cancer diagnosis and treatment[13–17]. There are many FDA approved liposomal formulations to treat cancer patient's i.e. liposomal doxorubicin Doxil® (Baxter Pharmaceuticals), vincristine Marqibo® (Talon, San Francisco, CA). DaunoXome (Daunorubicin citrate liposome injection), Onivyde (Irinotecan Liposome Injection), Dauxosomes, LEP-ETU (Liposome Entrapped Paclitaxel) etc [3, 5, 18].

Hypophyllanthin, a broad spectrum bioactive lignan found in the leaves of *Phyllanthus amarus Schum. & Thonn*[19–21]. Hypophyllanthin is a potent inhibitor of cytochrome p450 enzymes (Phase I enzymes). It exhibited antitumor activity in swiss albino mice and shown cytotoxic effects on K-562 cells[22–24]. This compound is highly insoluble in water. On oral route of administration, hypophyllanthin showed peak plasma concentrations up to 0.15–0.22 μg mL^{-1} within 1 hour and followed by a gradual decline to 0

after 24 hours. However, oral absorption of hypophyllanthin was incomplete and has poor AUC due to low aqueous solubility[25, 26]. This research focuses on developing hypophyllanthin loaded pegylated liposomes to overcome the poor aqueous solubility and improve the pharmacokinetic profile [27].

2. Materials

Hypophyllanthin was extracted and purified in-house. 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 donated by Lipoid, Germany. Cholesterol, pepsin, pancreatin, sodium taurocholate, mannitol, Tween 20, and stearic acid were procured from Sigma Aldrich, Germany. HPLC grade solvents such as 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

High-performance liquid chromatography (HPLC) was used to quantify the hypohyllanthin in the *in vitro* and *in vivo* samples [28]. The HPLC system consists of a quaternary pump (Waters Delta Prep HPLC system, USA), a manual injector (Rheodyne, Cotati, CA, USA) equipped with a 100µL sample loop and a variable wavelength programmable photo diode array (PDA) detector (Waters 2998 PDA, USA). The system was equipped with the EMPOWER 2 software (Waters, Milford, MA, USA) for data acquisition and processing. Analyses was performed on C-18 column {(250mm×4.6mm; particle size 5µm) (Waters symmetry)} with methanol: water (66:34 v/v) as mobile phase flowing at 1.0 ml min^{-1}. The injection volume of 20 µL and absorption wavelength (λ_{max}) of 225 nm was used.

3.1.1 Preparation of standard solutions

Hypophyllanthin stock solution was prepared by transferring 100 mg hypophyllanthin to a 100 mL volumetric flask containing 70 mL methanol. Then solution was sonicated for 10 minutes and finally made volume to 100 mL with methanol. A minimum of twelve standard solutions with concentrations ranging from 0.5 to 100 µg mL^{-1} were prepared by diluting the stock solution appropriately with the mobile phase. The sample concentration was estimated by external calibration.

3.1.2 Preparation of samples

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 during sample preparation, and the method produced a consistent response. Then, 500 µL of methanol was added to precipitate the proteins, which were 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 100 µL methanol and vortexed. The supernatant was separated and analyzed for drug content by the validated HPLC method. Samples were analyzed on the day of preparation and were stored at -20°C until the analysis.

3.2. Preparation of conventional and PEGylated liposomes

Table 1
Composition of different formulations (conventional and pegylated liposomes) tested in this study

Formulation	Hypophyllanthin (mg)	DSPC (mg)	Cholesterol (mg)	DSPE MPEG2000 (mg)	Stearic Acid (mg)	Ratio of Hypophyllanthin: DSPC: Cholesterol: DSPEMPEG2000: Stearic Acid
HPL1	2.5	60	40	0	10	1:24:12:0:4
HPL2	5	60	40	0	10	1:12:8:0:2
HPL3	10	60	40	0	10	1:6:4:0:1
HPL4	5	70	30	0	10	1:14:6:0:2
HPL5	10	70	30	0	10	1:7:3:0:1
HPL6	5	60	40	5	10	1:12:8:1:2
HPL7	5	60	40	10	10	1:12:8:2:2
HPL8	5	60	40	15	10	1:12:8:3:2

Film hydration technique was used to synthesize the conventional and pegylated liposomes by using DSPC, cholesterol and DSPE-MPEG2000. Stearic acid concentration was kept constant in all the formulations and was added to impart the charge on surface of the vesicles. As mentioned in the Table 1 the different ratios of hypophyllanthin, DSPC, cholesterol, stearic acid and DSPE-MPEG2000 were mixed and chloroform/methanol (9:1 v/v) was used to dissolve each composite in a round-bottomed flask. The rotary evaporator (Buchi Rotavapor, Switzerland) was then connected to the flask and before starting the vacuum pump the flask with rotary was rotated at a rate of 40 rpm at 65°C for 25 minutes. By applying a very low vacuum the chloroform/methanol mixture was completely evaporated which lead to form a thin lipid film on the walls of the flask. Next step is to hydrate the lipid film and was performed by using with 10 mL of phosphate-buffered saline (PBS; pH 7.4) and vortexed (Vortex mixer, Genei Mumbai) for one

hour at 65°C (approximately 10°C above the phase transition temperature (Tg) of DSPC). Large unilamellar vesicles were resulted and extruded 10 times through an extruder having 200 nm polycarbonate filter papers (Whatman, Denmark) at 65ºC above the Tg of DSPC to obtain small unilamellar vesicles (SUVs). Finally, the freeze drying was performed on the extruded SUV suspension to obtain the fine powder of liposomes.

3.2.1 Lyophilization cycle

Extruded liposomal suspension (5.0ml) was separated and placed in a 15 mL glass vials (Borosil, India) and freeze drying was performed by using 5% (w/v) mannitol as a cryoprotectant. Initially, the freeze dryer (Lyodel, India) was precooled to -40°C and later 0.5 ml mannitol solution was added to vials with liposomal formulation and then the temperature was decreased to -70°C at a rate of 0.5°C/min and the set temperature was held for 30 min. Then, for 10 hrs the chamber temperature and pressure were maintained at -16°C and 0.5 Torr, respectively. During secondary drying, the final temperature was set to + 20°C with a stepwise increment of -16°C, the holding temperature is for 2 hrs at each step. At last, after withdrawing the vials from the freeze dryer they were screw-crimped immediately[29–31].

3.2.2. Reconstitution of lyophilized liposomes

The lyophilized liposomes were reconstituted by gently shaking for 30 seconds in a phosphate-buffered saline (PBS; pH 7.4). After reconstitution, the appearance of the cake, vesicle size, size distribution (polydispersity index-PDI), encapsulation efficiency (EE), and *in vitro* drug release characterization was performed on freeze-dried formulations.

3.3. Evaluation of liposomes

The formulated liposomes were evaluated based on several parameters such as encapsulation efficiency (%EE), particle size, zeta potential, polydispersity index (PDI), X-ray diffraction (XRD), Fourier *transform infrared spectroscopy, transmission electron microscopy (TEM), differential scanning calorimetry and thermogravimetric analysis (DSC-TGA).* Other studies like stability, toxicity and *In vitro* drug release were also studied. All animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare.

3.2.1 Encapsulation efficiency (% EE) determination

The ultracentrifugation technique was used to determine the % EE of the liposomes. The liposome suspension of 2.0 ml was centrifuged at 25, 000 rpm at a controlled temperature of 4°C (Remi cooling centrifuge) for 2.0 hrs. The HPLC method as described in section 3.1 was used to determine free drug content that is withdrawn from supernatant. The % EE was estimated using Eq. 1. For each formulation, the procedure was repeated three times and the average of three replicates was reported.

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

3.2.2 Size, size distribution, polydispersity index, and zeta potential analysis

The liposomal suspension was diluted 10 times with 0.1 M sodium chloride solution. DLS (dynamic light scattering) (Nano partica, Horiba Scientific, Japan) was used to determine the size and size distribution of liposomes by taking the average of 3 measurements. Also, zeta potential and 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

Transmission electron microscopy (Hitachi H-7500, Japan) was used to examine the liposomal formulation size, shape, and surface layers. 20 µL of the sample was deposited over carbon-coated copper grids with 200 mesh for 60 s and dried. Excess liquid was blotted from the grid with filter paper to form a thin film and finally stained with 2% uranyl acetate, air-dried, and examined under a transmission electron microscope.

3.3.4 FTIR analysis

FT-IR spectra of pure hypophyllanthin and liposomal formulations were recorded on a Bruker Spectrophotometer (Switzerland). The samples of interest were prepared as KBr discs by applying 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 hypophyllanthin, 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. 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. Samples were scanned from 25°C to 400°C at a rate of 10°C min^{-1} under an N_2 purge. Thermographs of pure drug, blank formulations, and selected lyophilized liposomal formulations were recorded using SDT Q600 V20.9 Build 20, TA Instruments, USA.

3.3.6 XRD analysis

The powder X-ray diffraction pattern of hypophyllanthin and selected liposomes in lyophilized powder forms was analyzed in the 2θ angle range of 5 to 89.99 using an XRD instrument (PANalytical XPERT-PRO, Netherlands).

3.4. *In vitro* dissolution studies

Dialysis membrane method has been employed to evaluate *in vitro* drug release for pure drug and liposomal formulations. Dialysis membrane (Himedia, molecular weight cut off 12,000) was washed under running water for 6 hrs to remove glycerol. Subsequently, sulfur from the membrane was removed by treating 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 by using 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 release media contains 0.1% Tween 80 to solubilize the released drug. The beakers were set at slow constant stirring and equilibrated to 37 ± 0.5 ºC. The activated dialysis membrane was filled with test products, and both ends of the membrane were sealed and suspended in the release medium (0.1 N HCl for 2 hrs followed by pH 7.4 phosphate buffer for 22 hrs). 2 mL of release medium was withdrawn from the beakers at regular intervals to estimate the concentration of hypophyllanthin by HPLC method (section 3.1), and the % cumulative drug release was calculated. All drug release studies were conducted in triplicate.

3.5. Stability of liposomal formulations

Selected freeze dried conventional and pegylated liposomal formulations containing hypophyllanthin (HPL2 & HPL7) were evaluated for their stability in different simulated GIT fluids (SGF, pH 1.2 and SIF, pH 6.8). 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 at pH 6.8. To simulate the effect of bile salts, 3 mM sodium taurocholate was added to the SIF.

The hypophyllanthin conventional liposomes (HPL2) and pegylated liposomes (HPL7) were reconstituted in PBS (pH 7.4) before use. One milliliter 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 in SGF and and 6 hrs in SIF. Then equal volume of 0.1 M ice-cold sodium hydroxide was added to SGF and 0.1 M HCl to SIF to inhibit the enzyme activity. The samples were then analyzed for vesicle size, PDI, zeta potential, and % encapsulation efficiency as described in section 3.3[31].

3.6. Toxicity Studies

Swiss mice (female) weighing 20–25 g (Mahaveer Enterprises, Hyderabad, India) were randomly divided into three groups of six. All formulations were administered orally at a dose of 1000 mg of hypophyllanthin per kilogram body weight. Group I served as the control group and was administered drug-free pegylated liposomes. Groups II and III were administered conventional liposome formulation (HPL2) and pegylated liposome formulation (HPL7), respectively. After 14 days of administration, blood samples from the retro-orbital plexus were collected in K_2-EDTA microcentrifuge tubes. Blood samples were centrifuged at 3000 rpm for 5 min to separate plasma. The plasma was then analyzed for the levels of various markers. Nephrotoxicity was evaluated using the levels of Blood urea nitrogen (BUN), plasma urea, and plasma creatinine levels, while plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels to evaluate hepatotoxicity. Afore-mentioned biochemical parameters were determined by using an autoanalyzer (RM4000 Biochemical systems International, Italy) using diagnostic kits obtained from Span Diagnostics (Span Diagnostics Ltd., India).

3.7. Pharmacokinetic evaluation of hypophyllanthin liposomes

The pharmacokinetics studies were carried out in the Phytopharmacology laboratory, at the University college of Pharmaceutical Sciences, Andhra University which was approved by the institutional animal ethics committee and CPCSEA (Regd. No.516/PO/c/01/ CPCSEA) for experimentation on animals. The pure hypophyllanthin, hypophyllanthin Conventional liposomes (HPL2) and hypophyllanthin Pegylated liposomes (HPL7) were selected for pharmacokinetics study in sprague dawley rats. The selected formulations were administered orally at a dose equivalent to 5 mg per kg body weight of hypophyllanthin respectively, by oral gavage. The freeze-dried formulations were suitably reconstituted with saline to achieve the desired drug dose in 0.5 mL volume, which was administered to animals. The free hypophyllanthin was suspended in sodium CMC and administered to rats. The healthy Sprague dawley rats were acclimatized to standard environmental conditions (23 ± 2°C, 55 ± 5% relative humidity, 12 h light/dark cycle) for one week before administering the drug. The rats were divided into six groups of 4 each. Group I was administered pure hypophyllanthin, whereas groups II & III were administered reconstituted hypophyllanthin conventional liposomes (HPL2) and pegylated liposomes (HPL7), respectively at a dose of 5 mg/kg body weight. 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 the noncompartmental 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 (hr) plots using Kinetica software by applying a log-linear trapezoidal rule.

4. Results And Discussion

4.1. Evaluation of Physicochemical Properties of Liposomes

The liposomal formulations of hypophyllanthin were prepared successfully by the film hydration technique. Various physicochemical properties of liposomal formulations were assessed, and results were shown in Table 2.

Table 2
Physicochemical properties of conventional and pegylated liposomes.

Formulation	Size(nm)	%EE	PDI	Zeta Potential(mV)
HPL1	128 ± 3.38	84.83 ± 0.19	0.198 ± 0.013	-33.90 ± 1.20
HPL2	130 ± 3.38	82.39 ± 0.16	0.251 ± 0.007	-34.27 ± 2.21
HPL3	134 ± 4.33	61.88 ± 0.20	0.315 ± 0.011	-34.87 ± 1.31
HPL4	197 ± 3.84	78.88 ± 0.20	0.239 ± 0.009	-38.05 ± 2.06
HPL5	205 ± 3.06	75.29 ± 0.30	0.304 ± 0.006	-40.07 ± 2.45
HPL6	126 ± 2.91	81.87 ± 0.54	0.148 ± 0.016	-34.38 ± 2.43
HPL7	128 ± 4.06	81.52 ± 0.55	0.191 ± 0.008	-35.14 ± 1.76
HPL8	132 ± 2.96	82.62 ± 0.25	0.263 ± 0.008	-35.35 ± 1.15

The mean vesicle size of hypophyllanthin liposomes was found to be in the range of 126 ± 2.91 nm to 205 ± 3.06 nm and polydispersity index (PDI) in the range of 0.191 ± 0.08 to 0.315 ± 0.011. These results shows that the liposomal dispersion approached a monodisperse stable system and delivers the drug effectively, due to the large surface area of its nanosized vesicles. The zeta potential values of liposomes were found to be in the range of -33.90 ± 1.20 mV to -40.07 ± 2.45 mV, as listed in Table 2. The obtained zeta potential values indicate the presence of sufficient charge to inhibit the aggregation of vesicles. Excellent %EE has been achieved for various liposomal formulations, as listed in Table 2, which could be due to the sufficient fatty acid chain length of selected DPSCs.

4.2. Stability in simulated GI fluids

In vitro stability studies in simulated gastrointestinal fluids revealed that conventional liposomes (HPL2 & HPL2) were unstable in both SGF and SIF, as presented in Table 3. The excess hydrogen ions and bile salt monomers may disrupt vesicular structures. Furthermore, cholic and taurocholic acid in the gastrointestinal tract can also increase the permeability of liposomes, whereas pancreatic lipases tend to lyse liposomes[32]. This rupturing and lysing of the liposomes can account for the changes in particle size and encapsulation efficiency, which are indicative of the destabilization of the system.

Table 3
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
HPL2	SGF pH 1.2	136 ± 4.71	189 ± 4.24	-33.84 ± 1.35	-30.28 ± 2.34	81.24 ± 0.22	71.67 ± 0.24
	SIF pH 6.8	136 ± 4.71	158 ± 4.05	-33.84 ± 1.35	-31.42 ± 2.34	81.24 ± 0.22	74.86 ± 0.18
HPL7	SGF pH 1.2	134 ± 3.51	164 ± 3.24	-34.78 ± 1.76	-31.16 ± 3.65	79.45 ± 0.41	76.29 ± 0.25
	SIF pH 6.8	134 ± 3.51	140 ± 2.68	-34.78 ± 1.76	-31.54 ± 3.65	79.45 ± 0.41	77.64 ± 0.22

In contrast, pegylated liposomes (HPL7) were found to be stable in both media. The reason behind their stability could be the protective role of PEGylation, which prevents the exposure of phospholipids to the harsh conditions of the gastrointestinal tract, suggesting the robustness of the formulation[31]. Even previous studies [33, 34] confirm that PEGylation prevents the action of bile salts such as sodium taurocholate and other gastric enzymes, thereby resists rupturing of liposomes.

4.3. *In* Vitro dissolution by dialysis

Figure 1 shows the *in vitro* drug release profiles of liposomal formulations. 37.71% of the pure drug was released over 24 hours. Liposomal formulations, irrespective of their chemical composition released between 6% and 10% of the drug in the acid medium (pH 1.2) for the initial two hrs. A sudden spike in drug release rate was observed after membrane sack was moved to phosphate buffer (pH 7.4). Overall, the conventional liposomes released drug in the range of 60.73–68.51% whereas pegylated formulations showed 73.19–86.50% drug release over 24 hrs. Drug release profiles clearly indicates that both conventional and pegylated liposomal formulations released drug in slow and extended manner when compared to pure drug. In addition, pegylated liposomes shows higher percent of drug release than conventional liposomes, as evident from Fig. 1. The extended-release profiles of liposomal formulations can be attributed to increased stability due to the incorporation of cholesterol into liposomes.

As the ratio of DSPEMPEG2000 to lipid concentration increased (from HPL6 to HPL7), the rate of drug release from the vesicles increased, but a further increase in the DSPE-MPEG2000 concentration (15%) in PHL8 did not show any significant increase in the rate of drug release. An interesting release trend was observed for conventional liposomes, HPL3 and HPL5, where 62.48% of the drug was released at the 5th hour for PHL3 and 63.49% of the drug was released at the 4th hour for HPL5. These formulations contain a relatively higher drug content (10 mg), which might be a factor in the decreased stability of liposomes

leading to precipitation followed by burst release. Moreover, HPL5 was relatively less stable than HPL3 due to the higher lipid concentration.

Table 4
Correlation coefficient (r) values in the analysis of release data of conventional and pegylated liposomes containing phyllanthin (HPL1-HPL8)

Correlation Coefficient (r)								
Formulation	Zero-order	First-order	Higuchi	Peppas	Erosion	Ko ($mg \cdot hr^{-1}$)	K_1 (hr^{-1})	'n' in Peppas equation
HPL1	0.9423	0.9813	0.9839	0.9711	0.9659	2.757	0.0415	0.648
HPL2	0.9349	0.9788	0.9834	0.9670	0.9690	2.929	0.0461	0.728
HPL3	0.7556	0.7576	0.8792	0.7733	0.9209	3.026	0.0484	0.837
HPL4	0.9132	0.9581	0.9757	0.9455	0.9550	2.569	0.0368	0.691
HPL5	0.7036	0.6921	0.8414	0.7176	0.9033	2.901	0.0461	0.905
HPL6	0.8967	0.9529	0.9654	0.9386	0.9545	3.309	0.0576	0.725
HPL7	0.8939	0.9706	0.9644	0.9508	0.9592	3.927	0.0898	0.811
HPL8	0.8877	0.9680	0.9618	0.9476	0.9566	3.930	0.0898	0.833

The drug release data was analyzed using zero-order, first-order, Higuchi, Hixson-Crowell (erosion), and Peppa's 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 model indicates a non-Fickian diffusion mechanism for drug release from the hypophyllanthin-loaded liposome formulations. The results of the data analysis of drug release profiles are summarized in Table 4.

The formulations HPL2 (conventional) and HPL7 (pegylated) showed optimum particle size, better entrapment efficiency, and maximum drug release in a controlled manner. Hence, these two formulations were selected for further studies, such as *in vivo* drug release studies, stability studies, XRD, DSC-TGA, and FT-IR analysis.

4.4. Drug and excipient interactions

TEM, FT-IR, DSC-TGA, and X-ray diffraction analyses were conducted to identify the interactions between the drug and excipients in selected liposomal formulations (HPL2 and HPL7). If there is a physical or

chemical interaction of the drug with excipients, a significant deviation in pharmacokinetic and pharmacodynamic properties will be observed.

4.4.1. TEM analysis

Figure 2 shows the photomicrographs of liposomes of HPL2 and HPL7, which corresponds to the unilamellar structure of liposomes. The obtained size was in good agreement with that from the particle size analyzer. Also, the formulations are spherical in shape.

4.4.2. FTIR analysis

Hypohyllanthin has characteristic FT-IR absorption bands at 2902 cm^{-1}, 2916.63 cm^{-1}, 2981.9 cm^{-1}, 3000.75 cm^{-1} (C-H stretching, 1088.34 cm^{-1}, 1102.36 cm^{-1}, 1127.37 cm^{-1}, 1151.21 cm^{-1}, and 1185.42 cm^{-1} (C-O-C stretching), as shown in Fig. 3.

The FTIR spectrum of HPL2 has characteristic absorption bands at 2850.33 cm^{-1}, 2917.85 cm^{-1}, and 2983.94 cm^{-1} due to CH stretching and bands at 1081.41 cm^{-1}, 1140.41 cm^{-1}, and 1159.67 cm^{-1} due to C-O-C stretching. HPL7 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 the case of HPL2, the characteristic CH stretching band shifted to 2917.79 cm^{-1}, 2984.4 cm^{-1}, and C-O-C to 1081.23 cm^{-1}, with a reduction in intensity. In the case of loaded pegylated liposomes-HPL7, the characteristic CH stretching band shifted to 2917.70 $cm^{-1,}$ and C-O-C to 1083.38 cm^{-1}. The peaks in the IR spectra of the pure drug were present in the IR spectra of the liposomes, without disappearance and significant change in the position which concludes that hypophyllanthin is compatible with DSPC, cholesterol, stearic acid, and DSPC-MPEG2000.

4.4.3. XRD analysis

The XRD patterns of hypophyllanthin, blank formulation of conventional liposomes, DSPE MPEG2000, selected conventional (HPL2) and pegylated liposomes (HPL7) are shown in Fig. 4.

Sharp peaks at diffraction angles (2θ) of 7°, 16°, 19°, 23°, and 24° were observed in pure drug. The conventional and pegylated liposomes, showed 2Θ characteristics similar to those of pure drug, without a significant shift in 2Θ and reduction in peak intensities. XRD results suggests that hypophyllanthin is free from interaction with DSPC, cholesterol, stearic acid, and DSPE-MPEG2000.

4.4.4. DSC-TGA analysis

Hypophyllanthin (pure drug) has a small endothermic peak characteristic of melting at 130.64°C, as shown in Fig. 5, while an endothermic peak at 325.99°C indicates the degradation of the drug. The blank conventional formulation and DSPE-MPEG2000 have glass transition temperatures (Tg) of 64.27°C and 58.6°C, respectively. HPL2 has a Tg of 68.06°C, while HPL7 exhibited two endothermic peaks: one at 58.6°C, which is characteristic of DSPE-MPEG2000, and the other at 64.67°C, which is characteristic of other excipients. Both formulations (HPL2 and HPL7) exhibited a characteristic peak for hypophyllanthin

at 130.64°C. Hypophyllanthin showed a high degree of thermal stability in HPL2 and HPL7, an added value, which is evident from the lack of a degradation peak at approximately 325°C, as seen in the pure drug. As the melting points of the drug did not show any significant change in the thermograms, suggesting that the drug has not formed any inclusive complex with excipients and is free from any physical or chemical interaction.

4.5. Toxicity studies

The toxicity profile of the test products was studied using different toxicity markers. Table 5 summarizes the results of ALT, AST, creatinine, plasma urea, and BUN in plasma samples collected from the treated animal groups. There was no significant difference in the levels of toxic markers between control and treated groups. The safe toxicity range reported in previous studies for different hepato- and nephrotoxic parameters were 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) [31]. The concentrations of all toxicity markers in the treated groups were within the safe toxic range.

Table 5
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. HPL2	16.24 ± 2.89	49.95 ± 4.66	8.42 ± 2.25	19.14 ± 1.84	0.79 ± 0.12
III. HPL7	17.37 ± 2.95	54.42 ± 6.12	9.86 ± 3.14	18.27 ± 1.75	1.15 ± 0.25

4.6. Pharmacokinetic studies

The plasma drug concentration over a period for test formulations was shown in Figure 6, and associated pharmacokinetic parameters were listed in Table 6. The C_{max} and T_{max} values for conventional liposomal formulations Figure 6: Plasma drug concentration over time for test products were improved by a factor of 2 and PEGylated liposomal formulations improved by factor of 4, compared to pure drug, demonstrating the enhanced oral bioavailability of hypophyllanthin and tendency of liposomal formulations to release the drug in a controlled manner compared to the pure hypophyllanthin drug.

The $AUC_{0\rightarrow\infty}$ values for HPL2 and HPL7 were improved by 4- and 8-fold, respectively, compared to pure drug. Similarly, 5.4- and 11.2-fold increases in the $AUMC_{0-\infty}$ ($ng{\cdot}h\cdot mL^{-1}$) values for HPL2 and HPL7, respectively, were 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 the test products indicate that the systemic circulation time of hypophyllanthin was improved for the liposomal products compared to the pure drug, especially from pegylated liposomes

(HPL7). The values for clearance (Cl) and volume of distribution (Vz) were superior for liposomal formulations, especially the pegylated form, when compared to the pure drug.

Table 6
Pharmacokinetic Data of Test Products

Test products			
Pharmacokinetic parameter	Pure Phyllanthin 5 $mg \cdot kg^{-1}$	Conventional liposomes (PHL2)	Pegylated liposomes (PHL7)
C_{max} ($ng \cdot mL^{-1}$)	1350.08 ± 51.04	2446.5 ± 101.65	4554.71 ± 98.54
T_{max} (hrs)	1.07 ± 0.02	2.02 ± 0.05	4.14 ± 0.09
$AUC_{0-\infty}$($ng.\ h \cdot mL^{-1}$)	7354.42 ± 578.25	29222.4 ± 1951.84	58631.87 ± 2515.46
$AUMC_{0-\infty}$ ($ng.\ h \cdot mL^{-1}$)	51629.5 ± 4723.11	280852.7 ± 22868.24	575386.67 ± 30454.92
MRT (hrs)	6.99 ± 0.1	9.58 ± 0.15	9.81 ± 0.11
K_{el}^{-1} (hr)	-0.16 ± 0.001	-0.14 ± 0.002	-0.13 ± 0.004
$t_{1/2}$ (hrs)	4.32 ± 0.02	4.95 ± 0.08	5.33 ± 0.005
Cl ($mL\ h^{-1}$)	697.38 ± 56.03	204.08 ± 13.37	85.91 ± 3.71
V_z (mL)	4342.30 ± 332.11	1334.48 ± 70.17	579.5 ± 18.81

The *in vivo* pharmacokinetic studies revealed that pegylated liposomes have superior pharmacokinetics and is desired formulation 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 drug.

5. Conclusions

Film hydration method was employed to successfully develop hypophyllanthin-loaded liposomes. The poor aqueous solubility and oral bioavailability of hypophyllanthin has improved by formulating the drug as liposomes. The obtained size of liposomes for selected formulations (HPL2 & HPL7) is suitable for better oral absorption. In addition, the TEM images confirm that the developed liposomes are unilamelar nanosized spherical vesicles. The drug and excipients has minimal to no interaction which confirmed by XRD, FTIR and TGA-DSC studies. Evaluated toxicity markers are with in safe toxicity limits for liposomal formulations when administered orally. Liposomal formulations exhibited sustained release of the drug,

as supported by pharmacokinetic data. Overall, the PEGylated formulations have shown superior characteristics in terms of stability and physicochemical and pharmacokinetic properties. The enhanced oral bioavailability of liposomal formulation certainly effective in producing pharmacological activity at relatively lower doses compared to free drug improving the patient safety and compliance.

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 approved by the Institutional Ethical Committee (IAEC) of Regd. No. 516/01/A/ CPCSEA, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Ganga Rao Battu: supervision, funding acquisition.

Naga P. D. Boppana: Writing, review & editing.

Nagavendra Kommineni: Writing, review & editing.

All authors read and approved the final manuscript.

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Figures

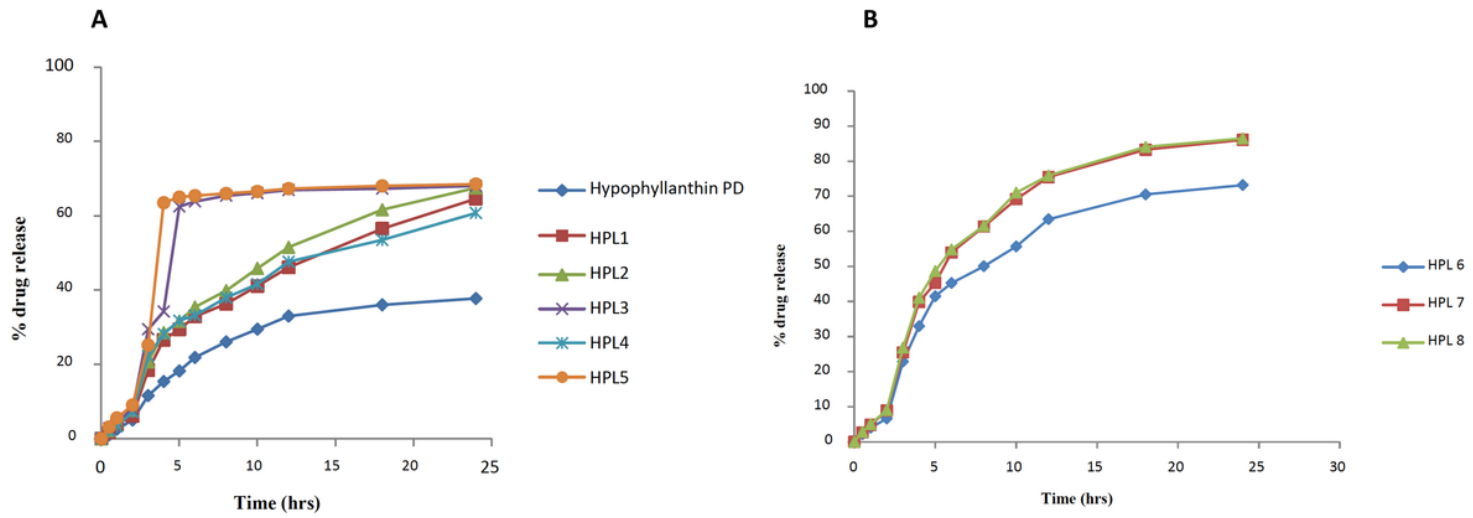

Figure 1

In vitro drug release profiles of test products. A: Pure hypophyllanthin and conventional liposomal formulations B: PEGylated liposomal formulations

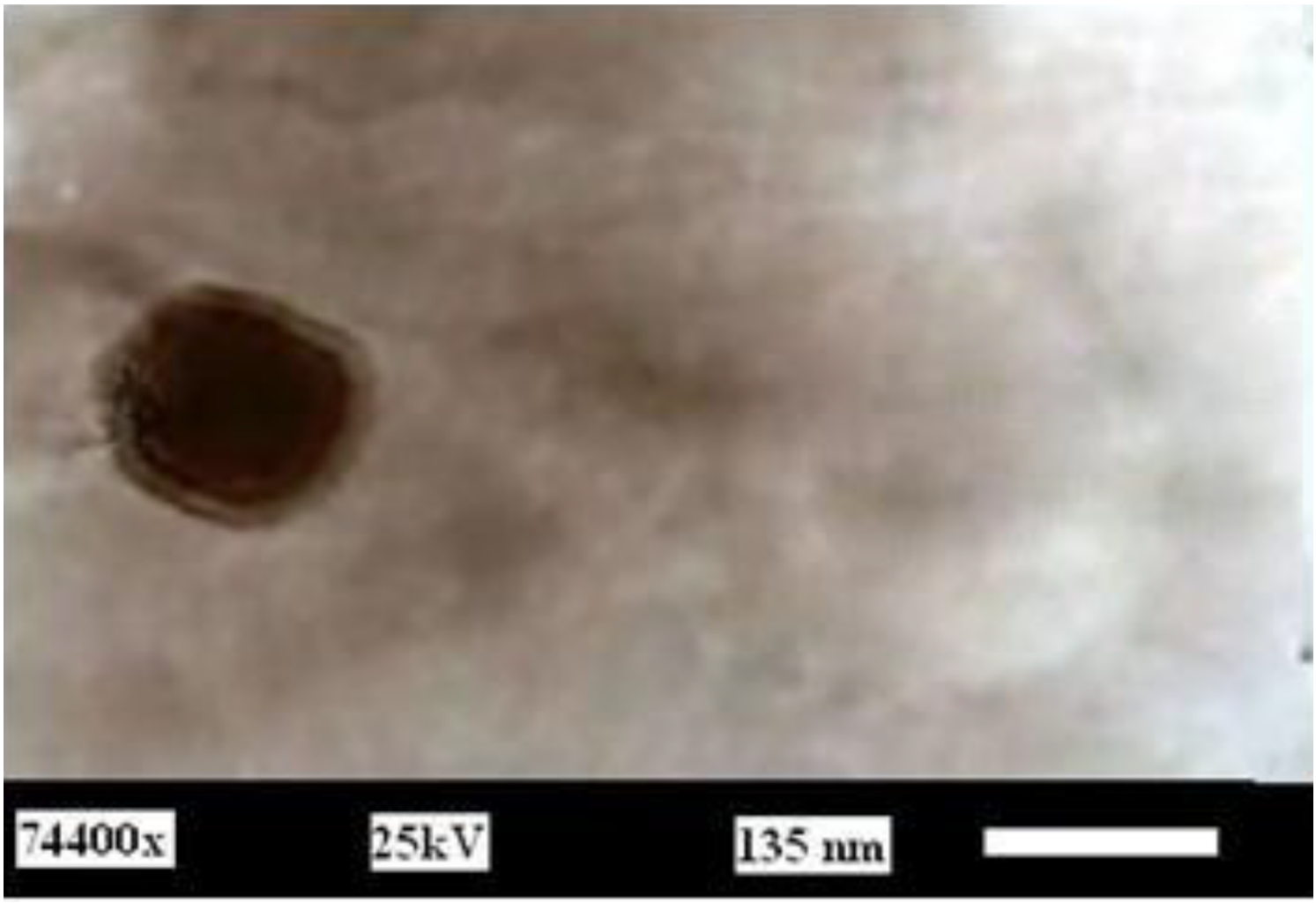

Figure 2

TEM image of HPL7

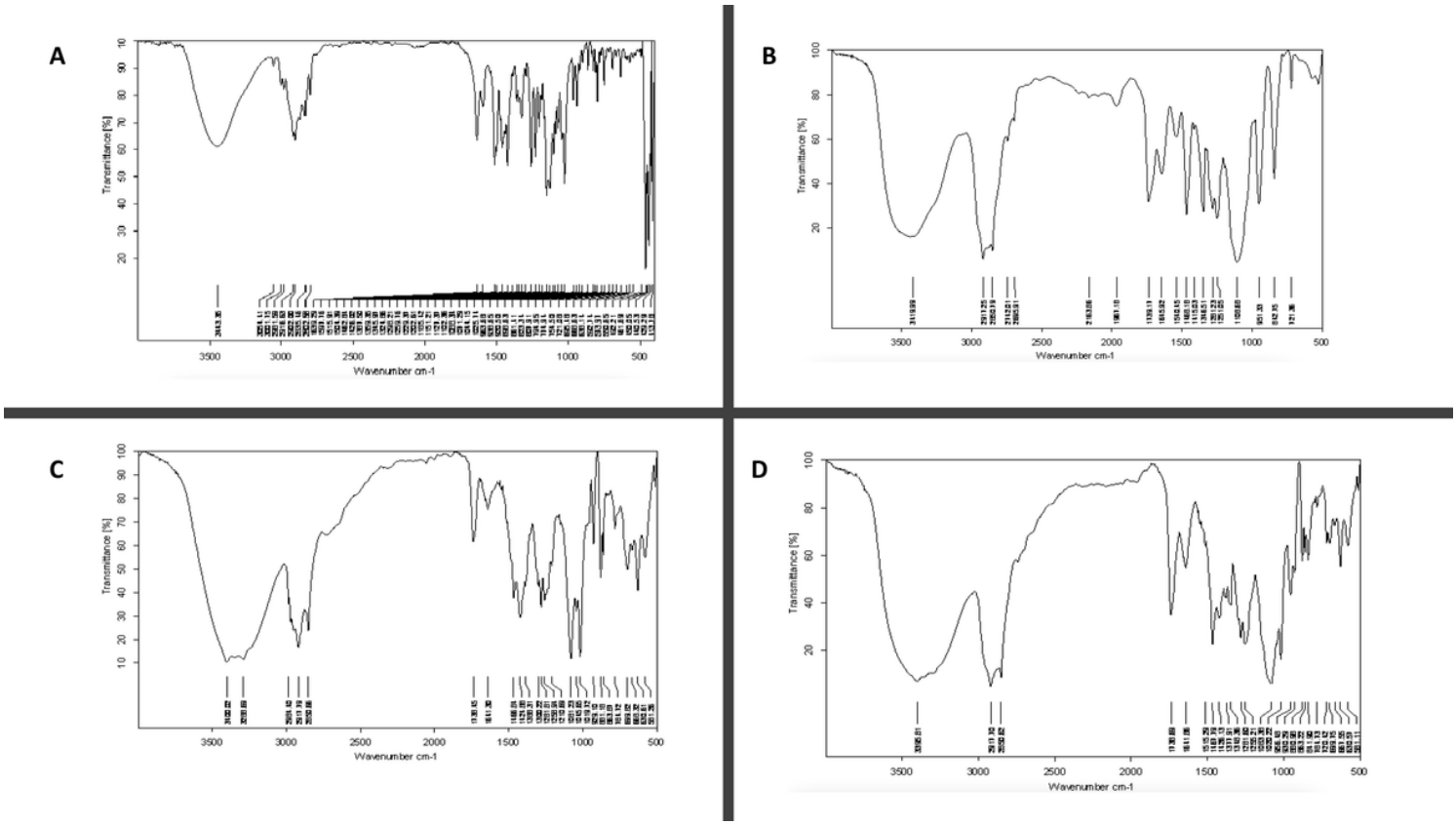

Figure 3

FTIR spectra of test products. A: Hypophyllanthin, B: DSPE:MPEG2000, C: HPL2, D: HPL7

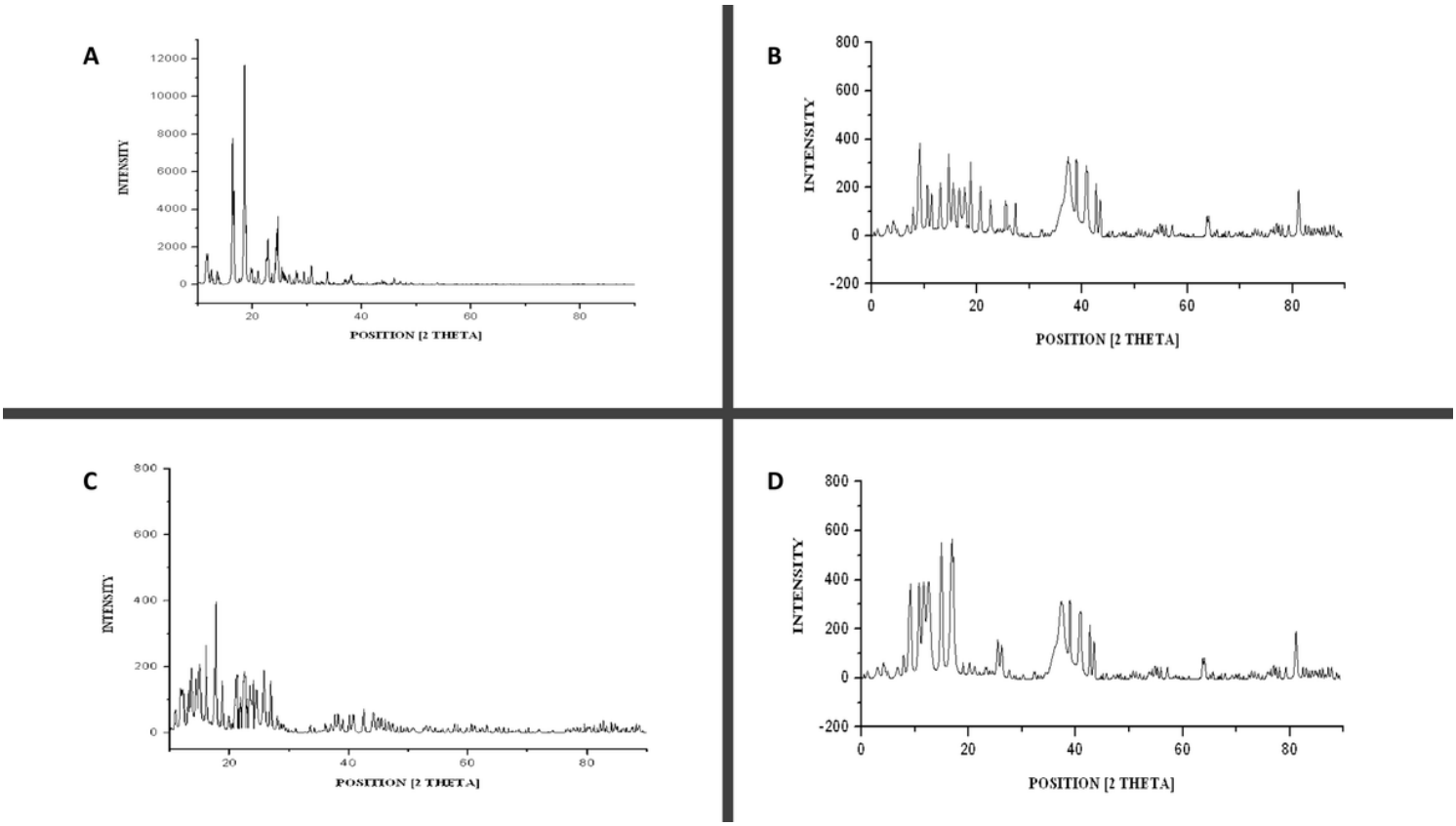

Figure 4

X-ray diffractograms of test products. A: Hypophyllanthin, B: DSPE:MPEG2000, C: HPL2, D: HPL7

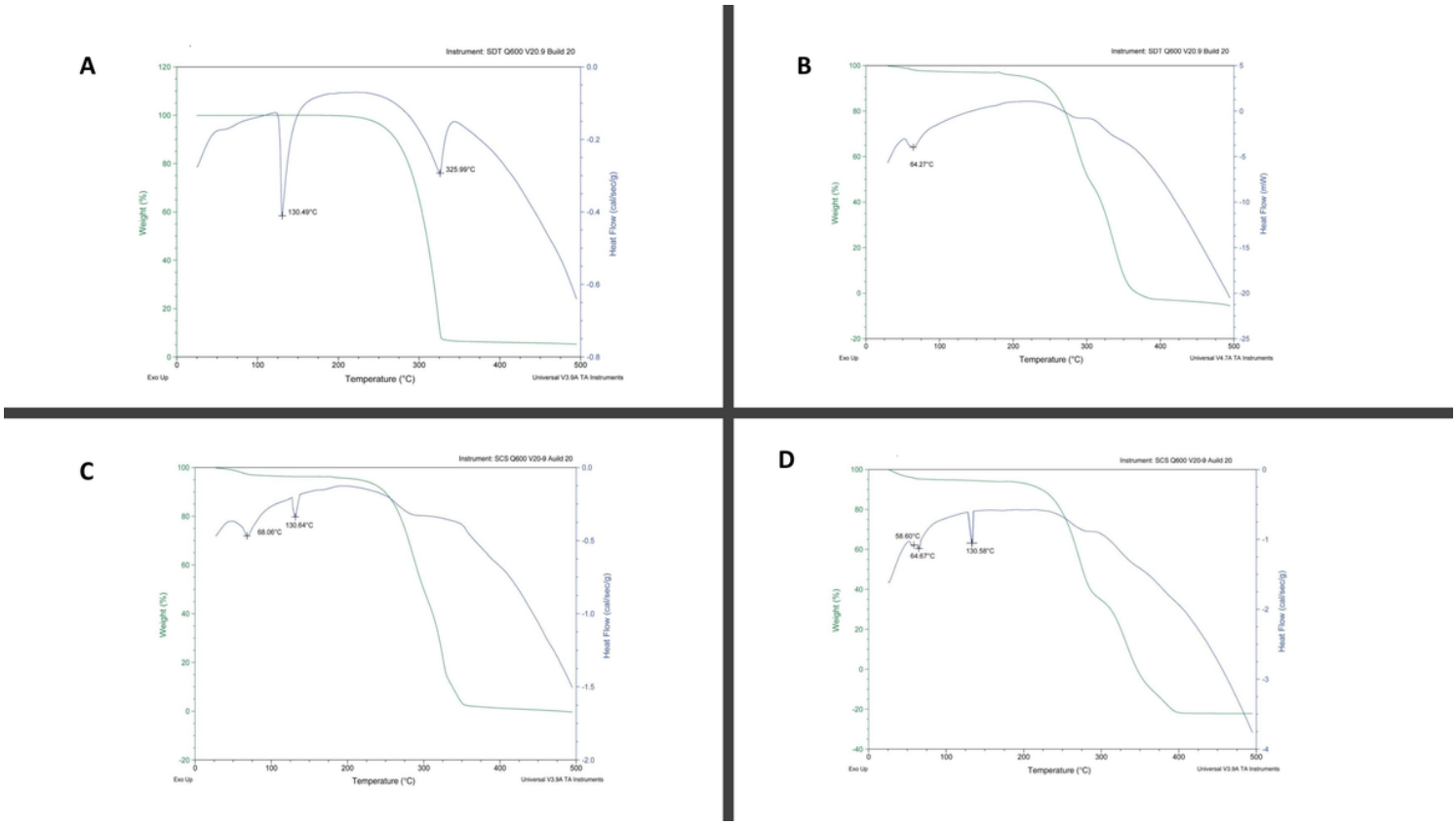

Figure 5

TGA-DSC thermal curves of the test products. A: Hypophyllanthin, B: Dummy PEGylated Formulations, C: HPL2, D: HPL7

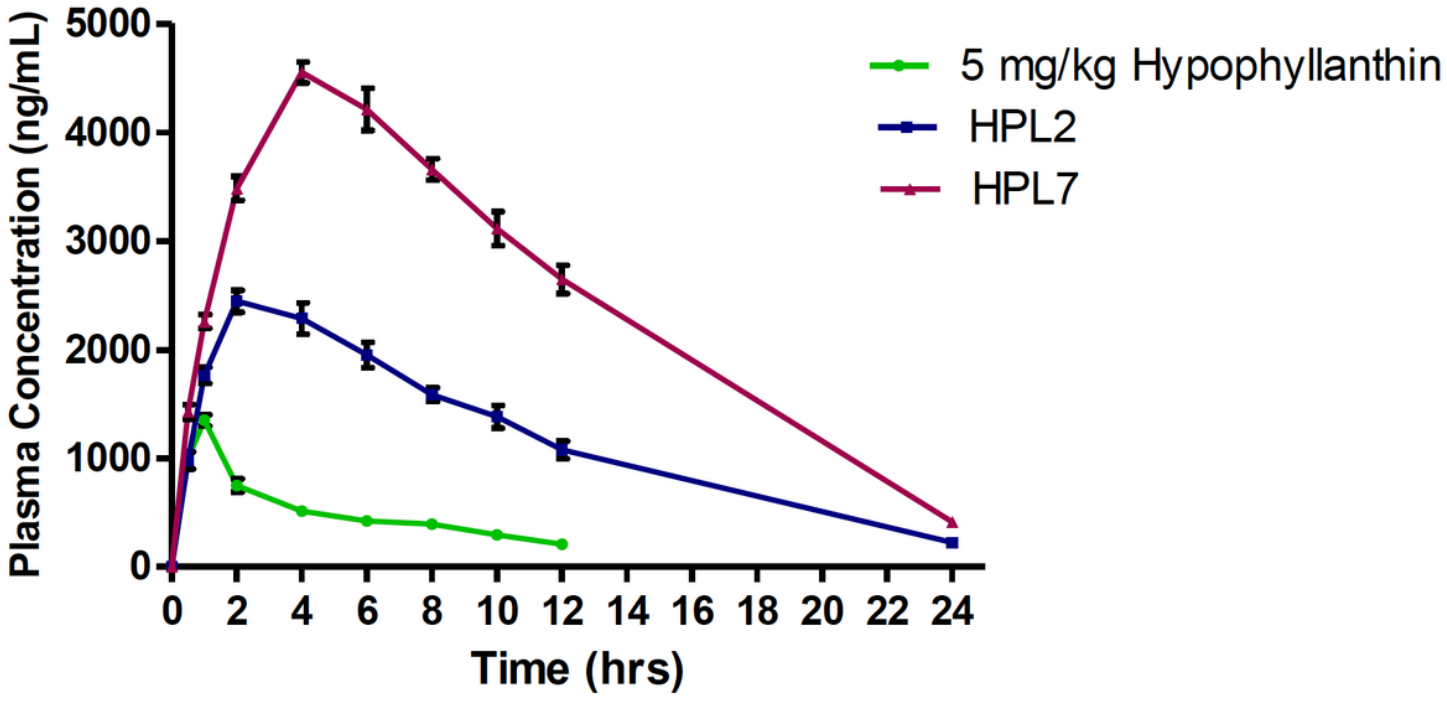

Figure 6

Plasma drug concentration over time for test products