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Formulation and evaluation of Phytosomes of Phyllanthus reticulatus

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Abstract:

The present study aimed to formulate and evaluate phytosomes of Phyllanthus reticulatus to enhance the bioavailability, stability, and therapeutic efficacy of its bioactive compounds. The aerial parts of P. reticulatus were collected, dried, and extracted using a hydroalcoholic solvent. The extract (HEPR) was subjected to in-vitro antioxidant evaluation using DPPH and hydroxyl radical scavenging assays, which demonstrated significant, concentration-dependent activity. HEPR-loaded phytosomes were prepared using the antisolvent precipitation method and optimized via Box–Behnken Design, considering the phospholipid:cholesterol ratio, temperature, and sonication time as critical formulation variables. The optimized phytosomes exhibited nanosized vesicles (157–253 nm) with high entrapment efficiency (55–73%), spherical morphology, and uniform dispersion. Physicochemical characterization using DLS, TEM, FTIR, and DSC confirmed successful incorporation of HEPR into the phospholipid matrix without chemical interactions. In-vitro release studies revealed sustained drug release compared to plain extract, highlighting the potential of phytosomal formulation in improving antioxidant delivery and therapeutic performance.

Keywords: Phyllanthus reticulates, Hydroalcoholic extract (HEPR), Phytosome, Antioxidant activity, Vesicle size, Entrapment efficiency, Box–Behnken design, Controlled drug release

Introduction:

Oxidative stress, caused by an imbalance between reactive oxygen species and endogenous antioxidants, contributes to the development of chronic diseases including cancer, cardiovascular disorders, and neurodegeneration. Medicinal plants are a promising source of natural antioxidants due to their bioactive compounds and minimal side effects. Phyllanthus reticulatus, widely distributed in tropical regions, is rich in flavonoids, phenolics, and tannins, which exhibit antioxidant, anti-inflammatory, and hepatoprotective properties.

Despite these benefits, the clinical application of P. reticulatus extract is limited by poor solubility, low bioavailability, and rapid metabolism. Phytosome technology, which involves complexation of

phytoconstituents with phospholipids, enhances absorption, stability, and controlled release of active compounds.

In this study, the hydroalcoholic extract of P. reticulatus (HEPR) was prepared. Subsequently, HEPR-loaded phytosomes were formulated, optimized using a Box–Behnken Design, and characterized for vesicle size, entrapment efficiency, morphology, physicochemical stability, and in-vitro drug release. This approach aimed to develop a novel phytosomal delivery system with improved antioxidant efficacy and potential therapeutic application.

Materials and method:

The aerial parts of Phyllanthus reticulatus were selected for the proposed study. The plant material was collected from local area. After collection, the plant parts were thoroughly cleaned to remove dirt and other foreign matter. The cleaned plant material was then dried under shade to prevent degradation of phytoconstituents and preserved for further use.

After drying in sunlight, the plant material was ground into a coarse powder and initially macerated with petroleum ether for defatting. The extraction process was continued until the material was completely free from fatty substances. Subsequently, an exhaustive extraction was carried out on 30 g of the dried aerial parts of Phyllanthus reticulatus using the maceration method with a hydroalcoholic solvent (ethanol: water, 70:30).

Formulation and Characterization of HEPR Phytosome

Design-Expert® software was utilized to optimize the formulation parameters and to investigate the effects of formulation variables on the phytosome characteristics. A Box–Behnken Design (BBD) was employed for the experimental investigation using Design-Expert® trial version 13.0.2.0 (Stat-Ease Inc., Minneapolis, USA).

The Box–Behnken design consisted of three factors at three levels, which were used to optimize the critical material attributes (CMAs) and evaluate their impact on the critical quality attributes (CQAs). The factor levels were coded as −1, 0, and +1, and the corresponding numerical values are presented in Table 3.1.

As shown in the table, the independent variables (CMAs) included the phospholipid:cholesterol ratio (X_1), temperature (X_2), and sonication time (X_3). The dependent variables (CQAs) selected for this study were vesicle size (Y_1) and entrapment efficiency (Y_2).

Direct response values obtained from different experimental runs showing the effect of Amount of Phospholipid: Cholesterol (X_1), Temperature (X_2), and Sonication Time (X_3) on critical quality attributes (CQAs).

Table: Effect of formulation and process variables on HEPR phytosome characteristics

Formulation Code	X_1	X_2	X_3
HEPR1	-1	-1	0
HEPR2	0	0	0
HEPR3	0	-1	1
HEPR4	0	0	0
HEPR5	0	-1	-1
HEPR6	0	1	-1
HEPR7	1	0	1
HEPR8	-1	0	1

HEPR9	0	0	0
HEPR10	-1	1	0
HEPR11	1	0	-1
HEPR12	0	0	0
HEPR13	0	1	1
HEPR14	-1	0	-1
HEPR15	1	-1	0
HEPR16	0	0	0
HEPR17	1	1	0

Characterization of HEPR-Loaded Phytosome

Determination of Entrapment Efficiency

High-performance liquid chromatography (HPLC) was employed to determine the entrapment efficiency (EE), defined as the amount of quercetin successfully incorporated into the phytosomal vesicles. The entrapment efficiency of HEPR-loaded phytosomes was determined using the centrifugation method.

Briefly, the phytosomal dispersion was centrifuged at 12,000 rpm at 4 °C to separate the entrapped drug from the unentrapped drug present in the supernatant. The transparent supernatant containing free quercetin was carefully removed. To lyse the phytosomal vesicles, 1 mL of 0.1% Triton X-100 was added to the pellet, followed by appropriate dilution using phosphate-buffered saline (PBS, pH 7.4).

The amount of quercetin present in the HEPR phytosome was quantified using HPLC at 280 nm (Singh et al., 2011). The percentage entrapment efficiency was calculated using the following formula:

$\%EE$=Amount of drug entrapped Total amount of drug added × 100

Physicochemical Characterization of HEPR Phytosome

The particle size, polydispersity index (PDI), and zeta potential (ZP) of HEPR-loaded phytosomes were measured using dynamic light scattering (DLS) on a particle size analyzer (Malvern Zeta Master ZEM 5002, Malvern, UK). The measurements were performed at 25 °C using the laser diffraction method, which detects backscattered laser light.

Zeta potential was determined by measuring the velocity and direction of nanoparticle movement under an applied electric field. The results were expressed as mean ± standard deviation. All measurements were performed in triplicate (n = 3) to ensure accuracy and reproducibility (Anwar et al., 2018; Wan et al., 2019).

Morphology of HEPR Phytosome

The surface morphology and shape of the optimized HEPR phytosome formulation were initially examined using an optical microscope (Cippon, Japan) equipped with a camera attachment (Minolta). A small quantity of the phytosome sample was placed on a glass slide and covered with a coverslip. The sample was observed under 10× magnification, and images were captured.

Further morphological evaluation and particle size analysis were conducted using transmission electron microscopy (TEM). A drop of the phytosome dispersion was placed on a carbon-coated copper grid and allowed to stand for 15 minutes. The sample was then negatively stained using 1% aqueous phosphotungstic acid and air-dried. The samples were visualized using a TEM

(Hitachi H-7500, Tokyo, Japan) (Yu et al., 2014).

FTIR Studies

(Mazumder et al., 2016; Macedo et al., 2021)

Fourier Transform Infrared (FTIR) spectroscopy was performed to evaluate the compatibility between HEPR and formulation excipients. KBr pellets were prepared by mixing the crude HEPR and optimized HEPR phytosomes with potassium bromide in a 1:100 ratio using a conventional pellet press technique.

The prepared pellets were placed in the IR chamber, and the spectra were recorded over a wavelength range of 4000–400 cm^{-1}. FTIR spectra were analyzed to identify characteristic functional groups and possible drug–excipient interactions.

DSC Studies

Differential Scanning Calorimetry (DSC) was used to study the thermal behavior of crude HEPR and HEPR-loaded phytosomes. Samples weighing 5–10 mg were sealed in flat-bottomed aluminum pans and heated at a constant rate of 10 °C/min over a temperature range of 50–400 °C under a nitrogen atmosphere (flow rate: 200 mL/min). Alumina was used as the reference standard (Rasaee et al., 2014; Sohail et al., 2016).

In-Vitro Drug Release Studies

The dialysis membrane technique was employed to study the in-vitro drug release behavior of plain HEPR solution and HEPR-loaded phytosomes. A known quantity of phytosomal formulation (equivalent to 1 mg/mL of HEPR) was placed inside a dialysis membrane, which was then immersed in a glass beaker containing 200 mL of 0.1 N HCl (pH 1.0).

The dissolution medium was maintained at 37 ± 0.5 °C with continuous stirring at 75 rpm. Aliquots of 2 mL were withdrawn at predetermined time intervals (1, 2, 4, 6, 8, 10, and 12 hours) and immediately replaced with fresh dissolution medium to maintain sink conditions.

The withdrawn samples were filtered using Whatman filter paper and analyzed by HPLC at 280 nm to quantify the amount of quercetin released. All experiments were conducted in triplicate. The drug release data were fitted to various kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models, to determine the release mechanism (Karole et al., 2019).

Results and discussion:

Fabrication and Evaluation of HEPR Phytosome

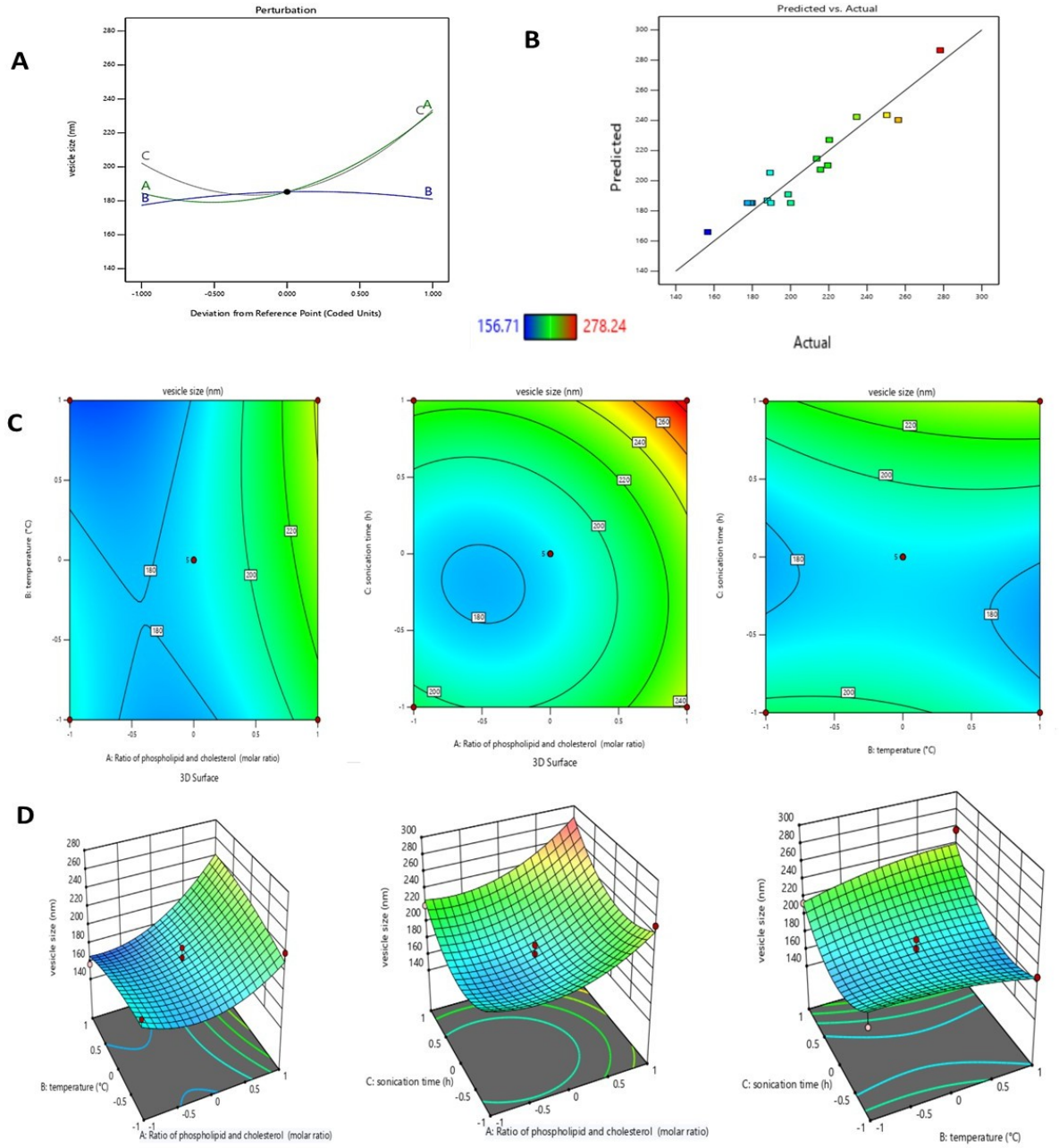
The antisolvent precipitation method was employed for the fabrication of HEPR phytosomes. The nanosized vesicular system was prepared using a hydroalcoholic extract of Phyllanthus reticulatus along with a phospholipid–cholesterol mixture. Phospholipids acted as the carrier, while cholesterol imparted rigidity to the vesicular system. The formulation parameters influencing phytosome characteristics included the phospholipid:cholesterol ratio, temperature, and sonication time. These variables were systematically evaluated using a Box–Behnken Design (BBD) (Marasini et al., 2012).

Box–Behnken Design for Optimization of HEPR Phytosome

Statistical optimization of HEPR phytosomes was performed using response surface methodology. A total of 17 experimental batches were prepared, and the obtained data were used to evaluate the effect of formulation variables on vesicle size and entrapment efficiency. Vesicle size

ranged from 157.73 ± 4.15 nm to 253.47 ± 6.63 nm, while entrapment efficiency varied from 55.23 ± 2.65% to 72.76 ± 1.77%. Regression analysis was carried out to understand the relationship between CMAs and CQAs,

Formulation code	**CQAs**			**CMAs**	
	X1	**X2**	**X3**	**Y1 (Vesiclesize, nm)**	**Y2 (Entrapment efficiency, %)**
HEPR1	-1	-1	0	195.74±8.93	60.93±3.91
HEPR2	0	0	0	183.37±7.92	66.8±2.50
HEPR3	0	-1	1	214.08±4.62	64.6±3.16
HEPR4	0	0	0	200.24±5.0	67.57±1.65
HEPR5	0	-1	-1	188.96±2.91	58.86±3.30
HEPR6	0	1	-1	187.3±4.42	60.4±6.91
HEPR7	1	0	1	274.8±7.82	69.8±1.76
HEPR8	-1	0	1	219.46±3.72	67.22±3.70
HEPR9	0	0	0	172.03±5.95	69.22±2.92
HEPR10	-1	1	0	157.73±4.15	62.49±2.50
HEPR11	1	0	-1	251.14±5.69	58.23±3.28
HEPR12	0	0	0	179.06±3.25	69.88±3.35
HEPR13	0	1	1	253.47±6.63	72.76±1.77
HEPR14	-1	0	-1	213.61±2.89	55.23±2.65
HEPR15	1	-1	0	219.11±3.36	61.13±4.48
HEPR16	0	0	0	180.36±5.32	66.7±1.18
HEPR17	1	1	0	238.92±4.14	72.27±1.50



Assessment of CMA Influence on CQAs

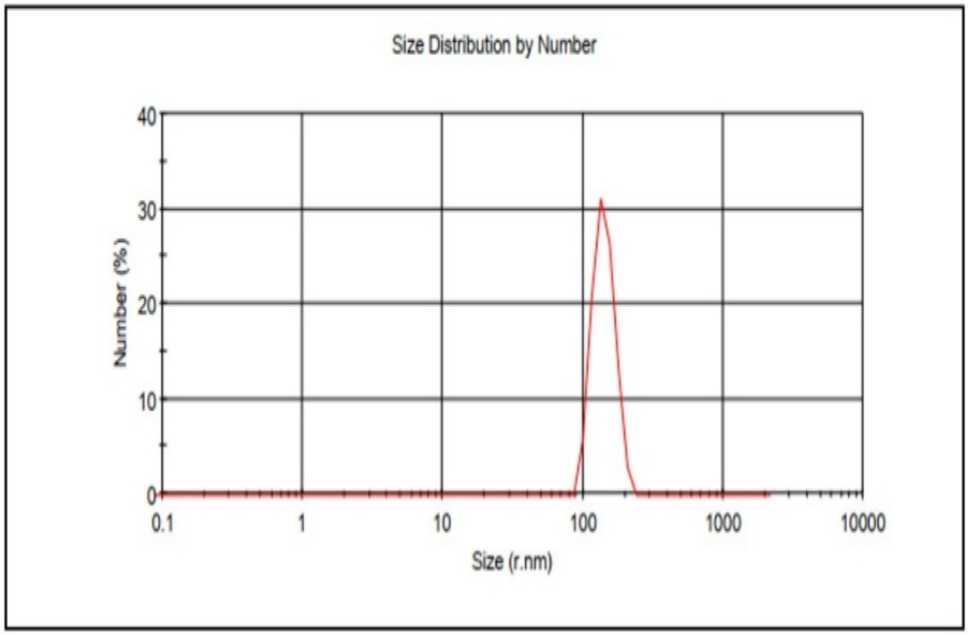
Effect on Vesicle Size

The vesicle size (Y_1) was significantly influenced by formulation variables. The developed polynomial equation indicated that the phospholipid:cholesterol ratio (X_1) and sonication time (X_3) had a positive effect on vesicle size, while temperature (X_2) showed a comparatively lower impact. An increase in lipid concentration and sonication time resulted in larger vesicles due to enhanced vesicle interaction and agglomeration. These effects were clearly depicted in the perturbation, 2D, and 3D response surface plots (Bouarab et al., 2014; Ahmed et al., 2019).

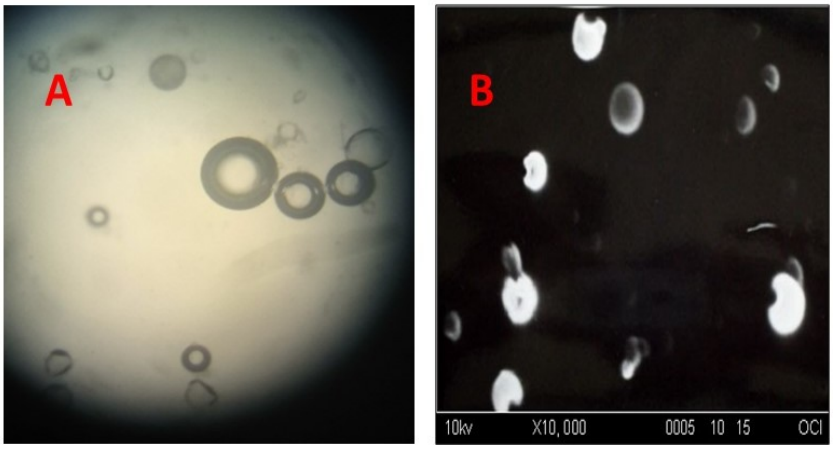
Effect on Entrapment Efficiency

The entrapment efficiency (Y_2) increased with higher levels of phospholipid concentration and sonication time, as represented by the coded polynomial equation generated using Design-Expert software. Increased phospholipid content enhanced drug-loading capacity, while longer sonication time promoted better vesicle formation and drug encapsulation. Temperature also played a supportive role in improving entrapment efficiency. The interaction effects were visualized through 2D and 3D response plots

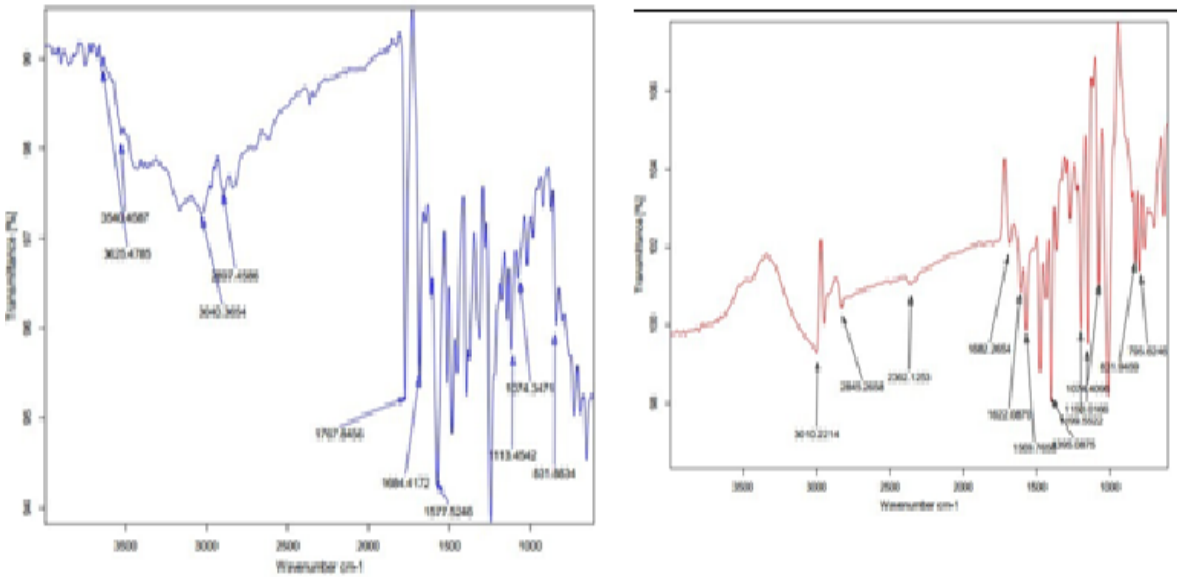
Results			Size (r.nm):	% Number	Width (r.nm):
Z-average (r.nm):	177.50	Peak 1:	177.50	100.0	33.47
Pdl:	0.145	Peak 2:	0.000	0.0	0.000
Intercept:	0.812	Peak 3:	0.000	0.0	0.000



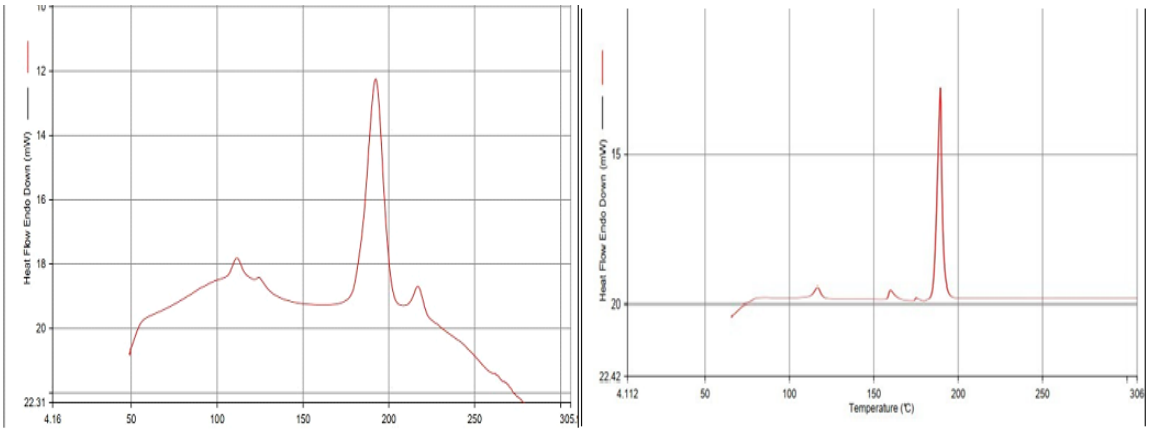
Particle size of optimized HEPR Phytosome



A) Surface morphology of HEPR Phytosome using Optical microscope, (B) TEM



FTIR Spectra of HEPR and HEPR Phytosomes



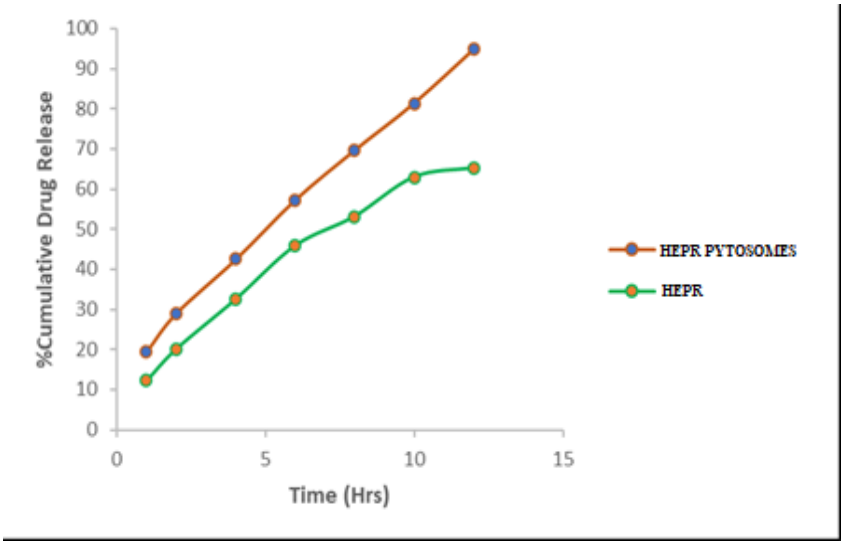
DSC thermogram of HEPR and HEPR Phytosomes

In-vitro dissolution study of phytosomes

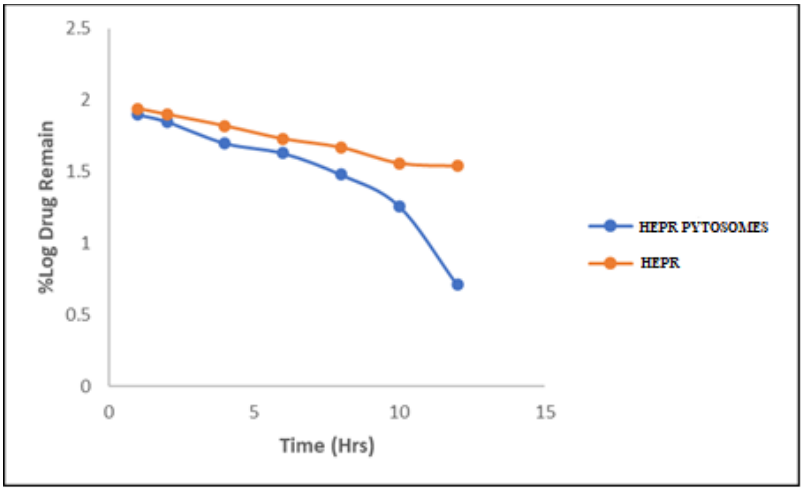
Time (hrs)	Sq.rt.of Time	Log Time	%Cumulative drug release of HEPR loaded phytosomes	%Cumulative drug release of HEPR
1	1	0.00	19.47±0.53	12.32±0.37
2	1.41	0.30	29.08±0.68	20.21±0.43
4	2	0.60	42.51±0.32	32.62±0.18
6	2.44	0.77	57.28±0.71	45.87±0.52
8	2.82	0.90	69.71±0.48	53.18±0.84
10	3.16	1.00	81.41±0.27	62.93±0.63
12	3.46	1.07	94.79±0.93	65.23±0.57

Log cumulative percentage drug release from HEPR loaded phytosomes and HEPR

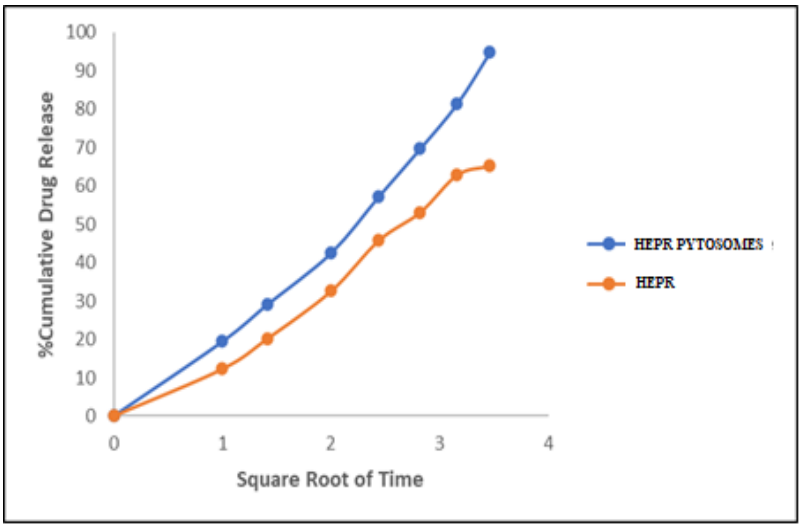
Time (hrs)	Sq.rt.of Time	Log Time	Log cumulative percentage drug release	
			HEPR loaded phytosomes	HEPR
1	1	0.00	1.28	1.09
2	1.41	0.30	1.46	1.30
4	2	0.60	1.62	1.51
6	2.44	0.77	1.75	1.66
8	2.82	0.90	1.84	1.72
10	3.16	1.00	1.91	1.79
12	3.46	1.07	1.97	1.81



In-vitro drug release profile of HEPR loaded phytosomes and HEPR to zero order release



In-vitro drug release profile of HEPR loaded phytosomes and HEPR according to first order release



In-vitro drug release profile of HEPR loaded phytosomes and HEPR according to Higuchi's model

Conclusion:

The present study was undertaken to evaluate the antioxidant potential of Phyllanthus reticulatus extract and to develop and characterize a phytosomal formulation (HEPR phytosome) for improved delivery and therapeutic performance. The hydroalcoholic extract of Phyllanthus reticulatus demonstrated significant in-vitro antioxidant activity, as evidenced by DPPH and hydroxyl radical scavenging assays. The antioxidant activity increased in a concentration-dependent manner, indicating the presence of bioactive phytoconstituents such as flavonoids and phenolic compounds, which are known for their free radical scavenging properties.

To enhance the bioavailability and stability of the extract, HEPR phytosomes were successfully formulated using the antisolvent precipitation technique. Optimization using the Box–Behnken design enabled systematic evaluation of the influence of critical material and process variables on vesicle size and entrapment efficiency. The optimized formulations exhibited nanosized vesicles with acceptable polydispersity and high entrapment efficiency, confirming the suitability of phospholipid–cholesterol combinations for phytosome development.

The vesicle size was significantly influenced by the phospholipid:cholesterol ratio and sonication time. Increased lipid concentration and prolonged sonication resulted in larger vesicles due to enhanced vesicle interaction and agglomeration. Entrapment efficiency improved with higher phospholipid content and sonication time, which can be attributed to increased lipid matrix availability and enhanced drug encapsulation capacity. These findings are in agreement with previously reported phytosomal and nanovesicular studies.

Physicochemical characterization confirmed the formation of stable phytosomes with uniform particle size distribution and sufficient surface charge to prevent aggregation. Morphological studies using optical microscopy and TEM revealed spherical and well-dispersed vesicles, supporting the DLS findings. FTIR and DSC analyses confirmed the compatibility of HEPR with excipients and suggested successful incorporation of the extract

within the phytosomal system without any chemical interaction.

In-vitro drug release studies demonstrated that HEPR-loaded phytosomes exhibited higher and sustained cumulative drug release compared to plain HEPR extract. The enhanced release profile may be attributed to improved solubility, increased surface area, and controlled release behavior provided by the phospholipid bilayer. Kinetic modeling indicated that the release pattern followed diffusion-controlled mechanisms, supporting the sustained release characteristics of the phytosomal formulation.

Overall, the findings of this study highlight that phytosomal encapsulation significantly improves the antioxidant potential, stability, and release behavior of Phyllanthus reticulatus extract. The optimized HEPR phytosome formulation shows promise as an effective natural antioxidant delivery system and may be further explored for in-vivo evaluation and therapeutic applications.

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