



Development and Characterization of Phytosomes for the Treatment of Anti-cancer Activity by Using the *Spirulina (Arthospira Plantesis)* Extract

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Abstract

The objective of this project is to develop and assess the anticancer activity of *Spirulina*. The current research is centred on obtaining a *Spirulina*-containing drug. The goal is to design and create Phytosomes, characterizing them based on relevant and crucial parameters. The solvent evaporation method was employed for the design of Phytosomes. Various parameters such as zeta potential, particle size, infra-red spectral analysis, % drug content, encapsulation efficiency, and % drug release (% DR) were evaluated. The optimized batch SP2 exhibited a zeta potential of -30.2mV and an average particle size of 554.2 nm is considered well-accepted and suitable due to its significant drug release. Notably, the In-Vitro drug release at the 12th hour and % Encapsulation efficiency of the optimized batch SP2 were reported as 90.87% and 90.42%, respectively. The optimized batch (SP2) underwent stability studies and In-Vitro anticancer assessments, confirming its acceptance, suitability, palatability, ease of administration, and overall elegance.

Keywords: *Spirulina*, solvent evaporation method, phytosomes, optimization, anticancer activity, stability study

INTRODUCTION

Herbal formulations consist of individual or combined herbal extracts used to treat various diseases. Due to the rising use of allopathic drugs, herbal remedies have lost their significance. Before introducing the allopathic system, traditional medicine has operated for over two thousand years. The effectiveness of plant-based traditional medicine is gaining attraction due to its cheap cost and minimum adverse effects. The whole plant extract is responsible for hepatoprotective, anti-ulcer, and anti-cancer properties [1, 2].

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Different allopathic medications are available in the market for treating a large number of diseases but are associated with various side effects. These drawbacks of allopathic medications can be overcome by use of herbal medicines. In addition, India had a very rich and diverse cultural history.

An important component of this culture and tradition is that of health and healing. Thus, there is a large health and healing related knowledge base present in all ethnic communities across the diverse ecosystems. However, over the last few centuries; this knowledge base has been diluted with increased influences from the main stream culture, which is derisive of local health

traditions. Variety of natural plant extracts and phytoconstituents possesses diverse medicinals. But their applications are limited because they having poor lipid solubility and destruct in gut [3].

In addition, stems also showed hepatoprotective, anti-pyretic, cytotoxic, anti-diabetic, and immunomodulatory properties. The roots have emetic, anti-stress, antioxidant, anti-ulcer, anti-diabetic, and visceral obstructions. The structure-based drug design is widely accepted for determining the binding affinity with specific proteins. Strong protein–ligand interaction indicates a high binding relationship between specific proteins and ligands. The molecular docking approach is applied to simulate a biomolecular interaction, and it provides data on the affinity of each ligand.

A lipid-compatible molecular complex between lipids and phytoconstituents is termed Phytosomes. It enhanced permeation and intestinal permeability compared to pure phytochemical extracts. It also has a preventive impact on herbal extracts by shielding them from digestive fluids and destroying gut flora. They may readily pass through the bilayer membrane and bloodstream. The lipids and nonionic surfactants generate unilamellar or bi-lamellar structures of Phytosomes. Spans and tweens have better entrapment and release characteristics among the nonionic surfactants [1, 2].

However, water soluble phytoconstituents (such as flavonoids, tannins, glycosidicaglyconeset) are poorly absorbed by passive diffusion, as result. They have the lipid-rich biological membranes and less bioavailability. Presently, various dosage formed of herbal formulation are available in the market but the main problem regarding herbal formulation is bio-availability. Herbal formulation has less bio-availability, without affection there safety parameter. Phytosomes is a new drug delivery system and prepared by complexing polyphenolic group phytoconstituents with phospholipids including phosphatidylcholine which bind molecules to each other on a cellular level. They have better bioavailability due to their capacity to penetrate the lipid rich bio-membranes and to protect the valuable constituents of the herbal extract from destruction by digestive secretions and gut bacteria. Phytosomes have the capacity to deliver the standardized plant extracts and phytoconstituents through several routes of drug administration which increases the bio-availability of the herbal formulation. Spirulina, blue green algae contains different phytoconstituents meant for the diverse biological activities. The extracts of this plant were prepared and evaluated for the cancer and other activities but main problem is less bioavailability. Therefore, the main objective of the present research is to develop *Spirulina* extract loaded Phytosomes with improved pharmacokinetic properties [3-5].

MATERIALS AND METHODOLOGY

Materials

Spirulina Extract are procured from GM Natural Foods, Islampur while Cholesterol and Chloroform were sourced from Fine Chemical, Mumbai. Phosphatidyl choline was purchased from Lipoid, Germany. All other chemicals employed in this study met the criteria for analytical-grade quality.

Preparation of Phytosomes by Solvent Evaporation Method

Spirulina (*Arthrospira platensis*) dry extract-containing Phytosomes were formulated through the reverse-phase solvent evaporation method. The compositions of various batches of Phytosomes preparations are presented in Table 1 [4].

Table 1. Composition of *Spirulina* Extract Phytosomes formulation Batches.

Ingredients	SP1	SP2	SP3	SP4	SP5
<i>Spirulina</i> extract (mg)	10	10	10	10	10
Acetone (ml)	5	5	5	5	5
n-hexane (ml)	5	10	15	20	25
Phospholipids (mg)	5	5	5	5	5

Anti-solvent Precipitation Process

The specified quantity of n-hexane (1 g) was dissolved in 5 ml of acetone and combined with a lipid mixture. Following this, 1 gram of Spirulina extract was introduced to the lipid-surfactant mixture and stirred magnetically at 500 rpm using a Remi magnetic stirrer 2MLH from Mumbai, India. Concurrently, 10 ml of phosphate buffer (pH 7.4) was gradually transferred into a round bottom flask at a rate of 1 ml/min. The resulting mixtures underwent reflux at 60°C–65°C for 1 hour with continuous stirring. Subsequently, a rotary flash evaporator, operating at 65°C and 50 rpm (Cyberlab Corporation, CR2001, Delhi, India), was employed to recover the organic solvent.

The aqueous phase was then homogenized at 8000 rpm for 2 hours at 4°C using a Remi homogenizer (RQT-1274, Mumbai, India) [9].

Finally, the phytosomes underwent sonication at 8°C with a probe sonicator (Citizen Digital ultrasonicator, CD4820) for 5 minutes at 30-second intervals. The formulated product was stored in an amber glass bottle at 2°C–8°C for further study.

CHARACTERIZATION OF FORMULATIONS

Drug Content

The 0.1 mL of prepared *Spirulina* extract Phytosomes was dissolved in 10 ml of methanol. This solution was analysed by UV-Visible spectrophotometer against methanol as a blank solution [10].

Particle Size and Poly-dispersity Index

The analysis of the particle size of Spirulina-loaded Phytosomes was conducted using a Malvern Zeta Size Analyser. The sample was placed in a cuvette, and subsequently, it was positioned within the Dynamic Light Scattering (DLS) analyser, where the particle sizes were measured and recorded [11].

Entrapment Efficiency

A centrifuge tube containing 4 mL of freshly prepared Phytosomes are inserted into the centrifuged machine at 15,000 RPM in a cooling centrifuge set to 40°C (REMI INSTRUMENT) for a duration of 30 minutes. Subsequently, the resulting Phytosomes formulation was centrifuged, and on each occasion, 2 mL of supernatant containing the drug suspension not encapsulated was separated and subjected to spectrophotometric analysis at a wavelength of 262 nm using a UV spectrophotometer (SHIMADZU UV-1900). As a control, a PBS with a pH of 6.8 was employed [12].

The Entrapment efficiency was calculated by using the formula,

$$\% \text{ Entrapment efficiency} = \frac{\text{Total amount of drug} - \text{Drug in supernatant}}{\text{Total amount of drug}} \times 100$$

Zeta Potential

Particle charge analysis were measured by using the Zetasizer instrument (HORIBA ZS 100) under controlled conditions at a temperature of 25°C. This analysis of the measurement of reflection of light source at an electrode voltage of 3.3 V, employing a combination of laser Doppler velocimetry and phase analysis. The viscosity of the dispersion medium was determined to be 0.896 mPas. The average zeta potential was calculated for each formulation based on three distinct samples [13].

Optical microscopy of Phytosomes

Optical microscopy were measured the structural behaviour of Phytosomes formulations. Optical microscopy pictures of Phytosomes formulations revealed characteristics such as lamellerity size, and shape. The samples were examined at a 10 X [14].

In-Vitro Drug Release Study

These experiments were conducted utilizing Franz diffusion cells. In the donor compartment, we introduced varying quantities of phytosomal formulations, each containing a consistent weight of 4 mg.

The receptor medium, located in the receptor compartment, consisted of a phosphate buffer solution with a pH of 7.4.

A semi-permeable cellulose dialyzing membrane with a molecular weight cut-off range of 12,000 to 14,000 Daltons was used to generate a distinct separation between the donor and receptor compartments. The membrane was pre-soaked in PBS with a pH of 7.2 for 24 hours before being used.

Throughout the experiment, the medium in the receptor compartment was constantly agitated by a magnetic bar rotating at a pace of 50 revolutions per minute. Throughout the 8-hour period, the temperature was strictly maintained at 37°C. To achieve ideal dissolving conditions, we withdrew 3 mL samples and replaced them with fresh PBS solution on a regular basis. Following this procedure, the collected samples were suitably diluted and subjected to analysis for drug release using a UV/visible spectrophotometer [15].

FTIR Study

FTIR spectra were gathered for the pure drug, excipients, physical mixture, and the optimized phytosomal formulation utilizing the BRUKER Alpha II FTIR Spectrophotometer equipped with a probe. Approximately 5 mg of each sample was introduced and spectral data were acquired within the range of 400-4000 cm^{-1} [16].

Stability Study

Physical stability of the Phytosomes solution was assessed over a two-month period. These formulations were stored in 10 ml coloured glass vials and refrigerated at a temperature of $4\pm 2^\circ\text{C}$.

Each month, samples were taken from each batch and analysed spectrophotometrically to assess the quantity of *Spirulina* that remained. The Zetasizer NanoZS device from HORIBA device, Japan, was also used to monitor changes in the size and size distribution of phytosome particles [12]. A visual assessment of selected phytosomal formulations for evidence of sedimentation and colour changes was also included of the experiment.

RESULTS AND DISCUSSIONS

Particle Size, Zeta potential and Entrapment Efficiency of Formulated Batches

The formed Phytosomes exhibit mono-disparity and uniform size, with their nanometer dimensions suggesting enhanced accumulation at the tumour site through the Enhanced Permeability and Retention (EPR) effect.

Zeta potential analysis, conducted using a Zetasizer, revealed that the particle size of Spirulina-loaded Phytosomes was determined to be in the nanometer range, (Figure 2) with a recorded value of -30.8 mV, as presented in Table 2 [2, 8].

Notably, formulation SP2, characterized by a drug-to-polymer ratio of 1:1.5 and a lipid concentration of 10 mg w/v IPM, demonstrated a remarkably high entrapment efficiency of 80.14%. It is important to emphasize that as the globular size increased across all formulations, the entrapment efficiencies of the phytosomal formulations also increased, [Fig. 1] particularly with higher lipid concentrations. These findings provide substantial evidence that elevating the drug-to-polymer ratio led to a significant enhancement in drug entrapment efficiency, attributed to increased viscosity [12].

Optical Microscopy of Phytosomes

The Phytosomes were observed to have a white appearance, and among the formulations, Phytosomes stood out as the most translucent. This transparency is attributed to the incorporation of both ethanol and lipid in the formulation composition [10] (Figure 3 A).

In Transmission Electron Microscopy imaging, Spirulina served as the negative stain for these samples. Spirulina, being an electron-dense and opaque reagent for electrons, effectively interacted with the vesicles. This interaction facilitated the visualization and differentiation of the membrane and lamellae of the vesicles, with phytosomes exhibiting particularly distinct features (Figure 3 B).

Table 2. Characterization of Particle size, Zeta potential and Entrapment efficiency of phytosomal batches are as -

Formulation Batches	Particle Size (nm)	Zeta Potential (mv)	Polydispersity Index	Entrapment Efficiency (%)
SP1	419.8	-20.8	0.327	87.91
SP2	554.2	-30.8	0.496	93.33
SP3	613.8	-28.3	0.578	90.43
SP4	602.4	-27.9	0.321	88.12
SP5	403.9	-21.9	0.789	87.39

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	451.4 nm	135.7 nm	381.1 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	451.4 nm	135.7 nm	381.1 nm

Cumulant Operations

Z-Average : 552.4 nm
PI : 0.496

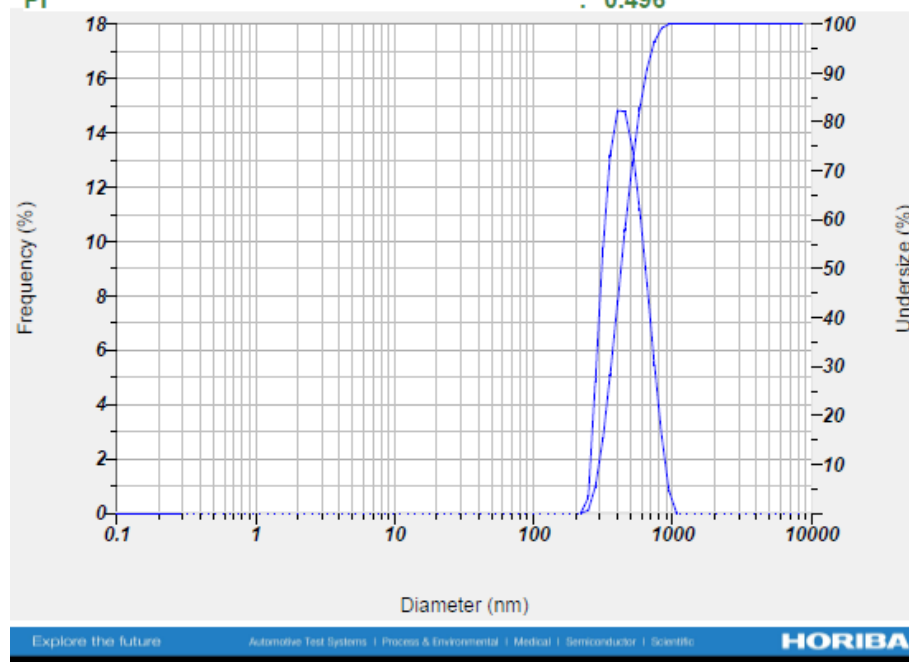


Figure 1. Particle size of optimized batch of phytosomal formulation (SP2).

FTIR Study

FTIR (Fourier Transform Infrared) analysis is a potent method for structural examination, revealing distinct characteristics in terms of band number, position, shape, and intensity for different functional groups. To validate the formation of phyto-phospholipid complexes, one can compare the spectroscopy of these complexes to that of physical mixtures. It's important to note that distinct studies may yield varied results. In the case of prepared phyto-phospholipid complexes containing rutin, the FTIR spectrum of a physical mixture of rutin and phyto-phospholipid complexes (Figure 5) closely resembled that of pure rutin. However, when examining Spirulina-phytosome complexes, the FTIR spectrum of

the phytosome complex displayed distinct peaks differing from those observed in sangria, phospholipids, cholesterol (Figure 6) and their mechanical mixtures (Figure 7). Additionally, an FTIR spectrum of pure Spirulina was obtained for reference [3, 6] (Figure 4).

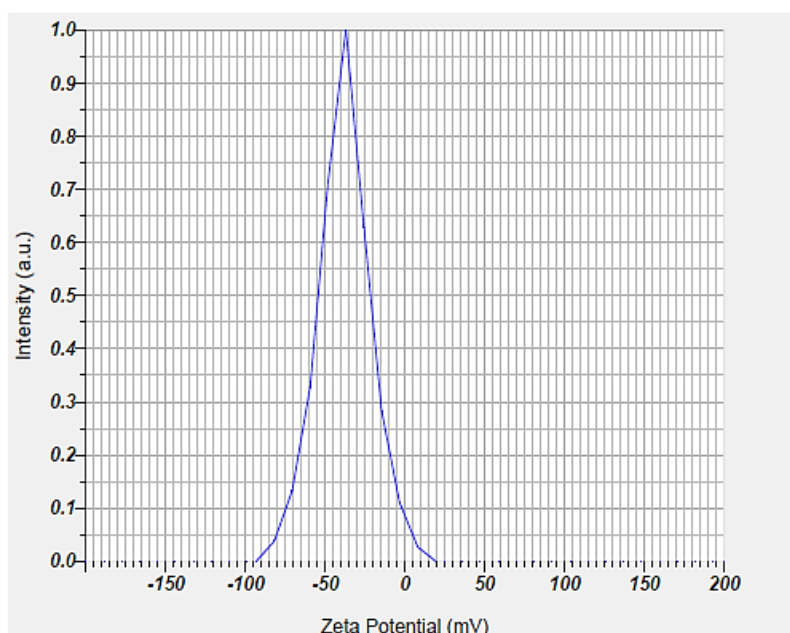
***In-vitro* Drug Release Study**

In-vitro percentage drug release (% DR) studies for all Phytosome formulations were conducted and are presented in the Table 3. The study spanned 12 hours, and the percentage of drug release was calculated at various time intervals. Notably, all batches demonstrated effective controlled release. A distinct relationship was observed between the polymer concentration and the rate of drug release from the Phytosomes. There was an inverse correlation between polymer content and the drug release rate from the formulated Phytosomes[7].

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-38.0 mV	-0.000196 cm ² /Vs
2	-- mV	-- cm ² /Vs
3	-- mV	-- cm ² /Vs

Zeta Potential (Mean) : -38.0 mV
Electrophoretic Mobility Mean : -0.000196 cm²/Vs



1/1

Figure 2. Zeta potential of Optimized batch of phytosomal batch (SP2).

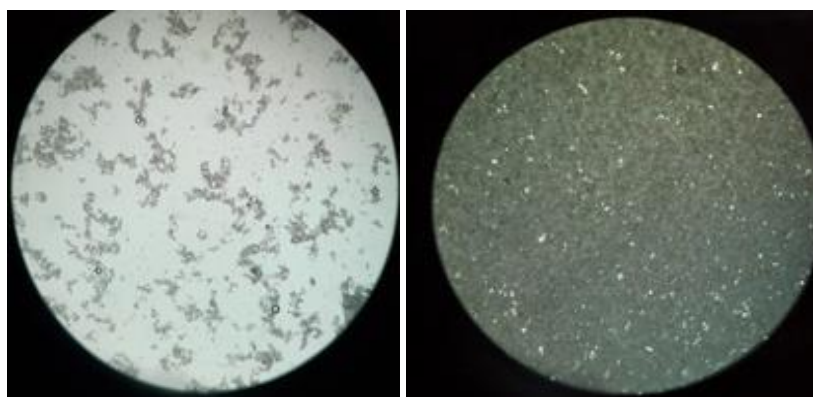


Figure 3. Optical microscopy of Spirulina Loaded Phytosomes (a) Before HPH & (b) After HPH.

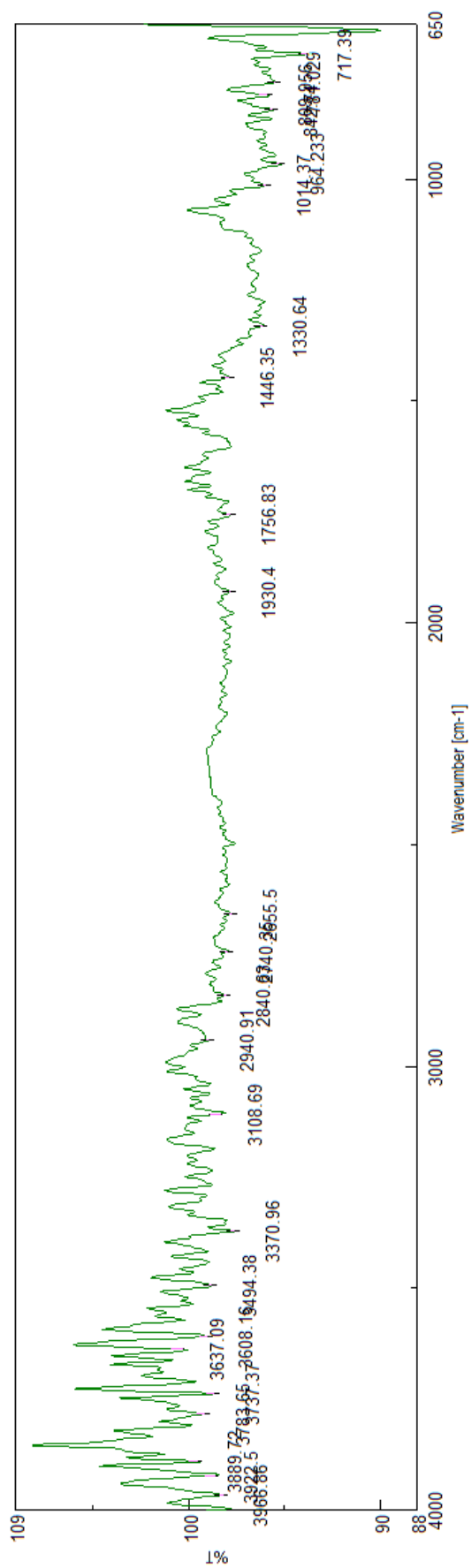


Figure 4. Pure spirulina extract.

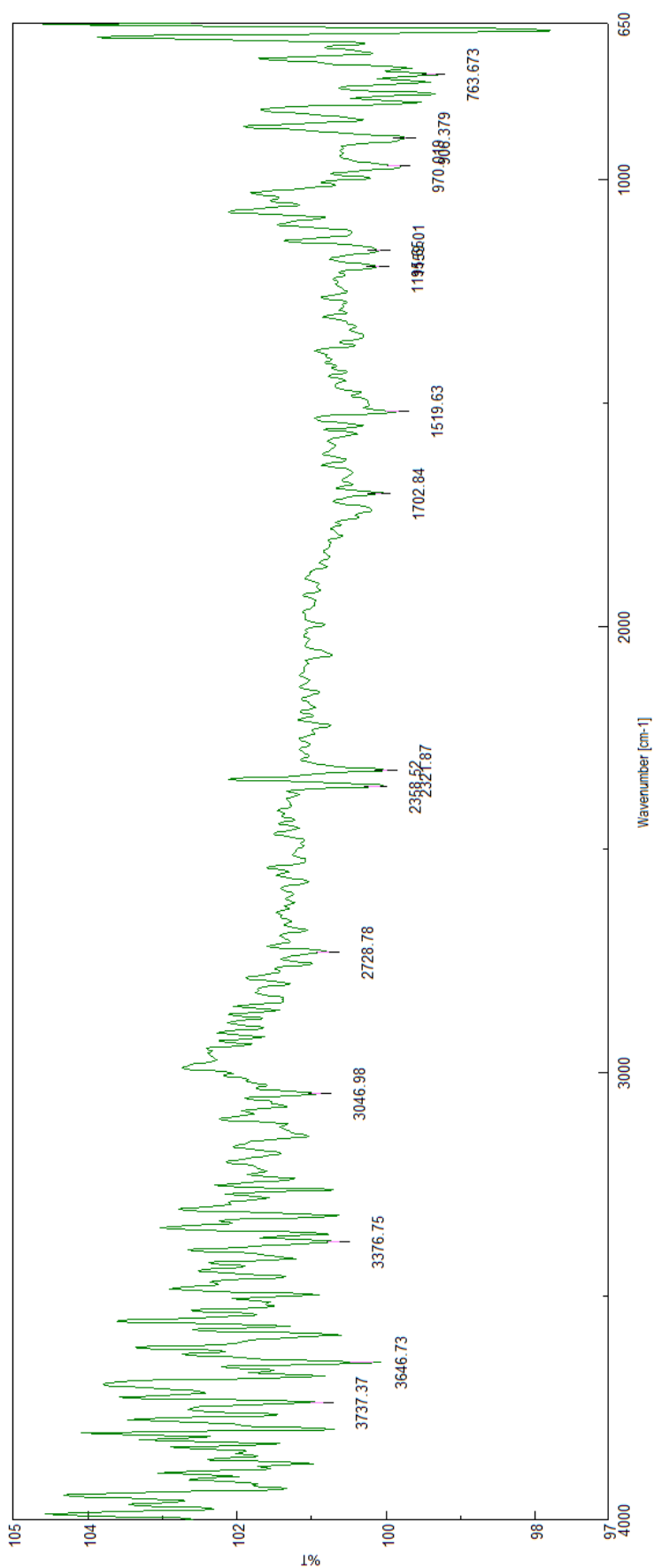


Figure 5. Phosphatidyl choline.

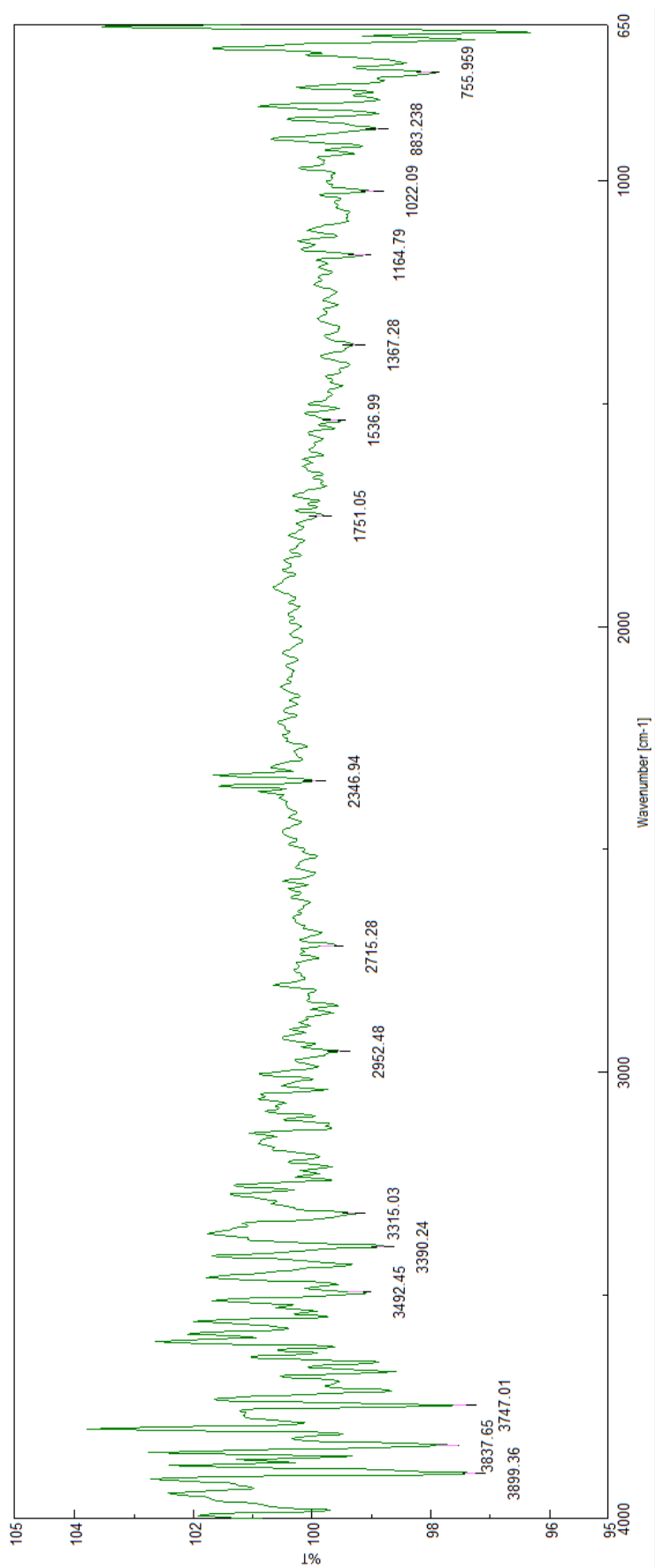


Figure 6. Cholesterol.

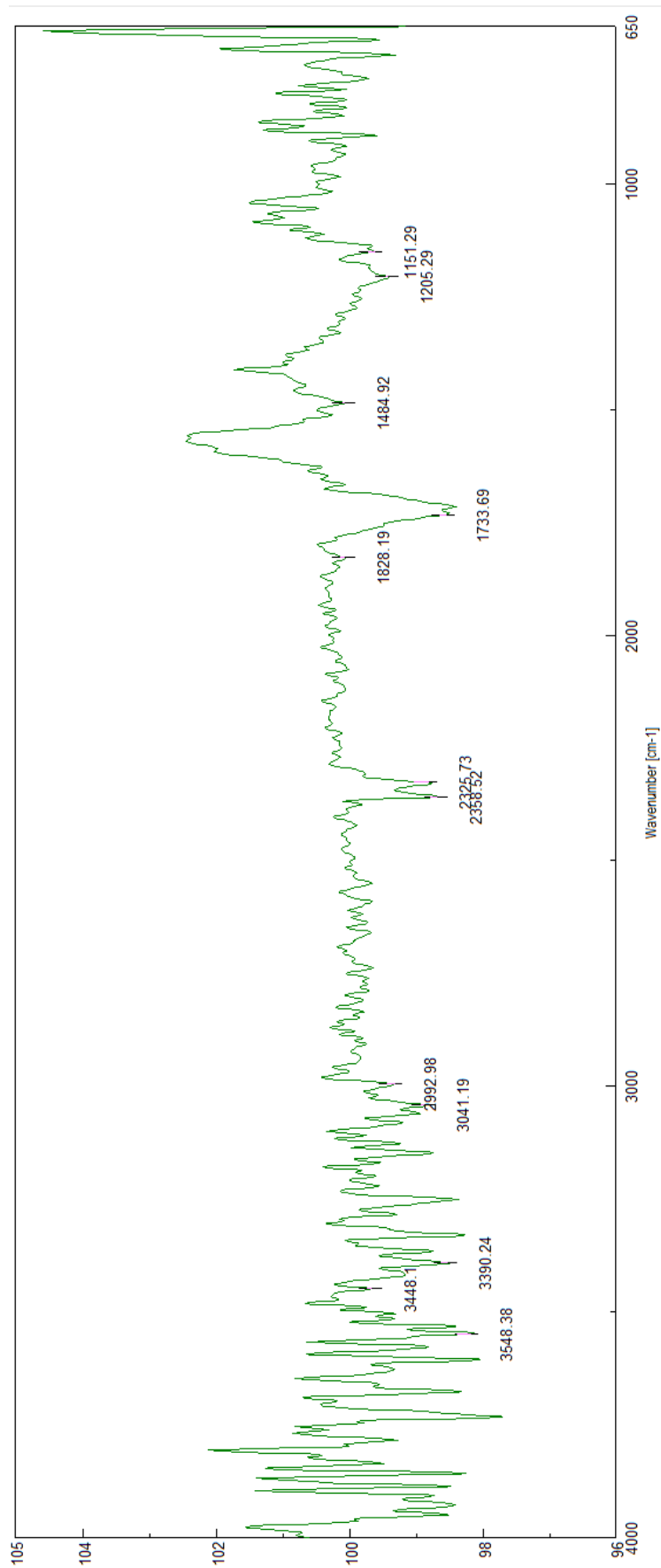


Figure 7. Spirulina, phosphatidyl choline, cholesterol and formulated batches.

In all Phytosome cases, it was observed that formulations with lower polymer content exhibited faster drug release, while those with higher polymer concentrations demonstrated a comparatively slower drug release profile. Across all batches, the drug release patterns fell within the range of $69.76 \pm 0.33\%$ to $89.79 \pm 0.11\%$ over the 12-hour course of the study. It's noteworthy that SP3 exhibited a higher drug release percentage of $90.87 \pm 0.35\%$ [9, 10].

Stability Study

The particle size was used to determine the stability of the final phytosomal formulation (SP2) and the percentage of drug entrapment before and after three months of refrigerated storage. Table [4] indicates that there were no notable alterations in particle size or zeta potential following three months of refrigerated storage at $4 \pm 2^\circ\text{C}$. In terms of drug entrapment within the phytosomal formulations, it was observed that drug leakage accounted for less than 15% of the initially entrapped Spirulina amount. At the end of the stability study period, all phytosomal formulations remained unchanged [12].

Trypan Blue Dye Exclusion Assay

The blue drill exclusion test is the most used test for cell viability. In this assay, cells are washed with HBSS (Hank's Buffered Salt Solution) and centrifuged for 10-15 minutes at 10,000 rpm. The process is repeated three times. The cells are suspended in a known amount of HBSS and the cell number is adjusted to 2×10^6 cells / ml. The cell suspension is dispensed into Eppendorf tubes (0.1 ml containing 2 litre cells). The cells are exposed to drug dilutions and incubated at 37°C for 3 hours. After 3 hours, the dye exclusion test, i.e., equal quality of the treated cells with the drug is mixed with Trypan blue (0.4%) and left for 1 minute. It is then loaded onto a haemocytometer and a viable and non-viable measurement is recorded within 2 minutes (Table 5).

Table 3. In-vitro drug release study of phytosomal batches of Spirulina extract.

S.N.	Batch Code	% Drug release
1	SP1	69.76
2	SP2	90.87
3	SP3	79.42
4	SP4	75.50
5	SP5	89.79

Table 4. Stability study of phytosomal formulated batches.

Formulation Batches	Particle Size (nm)	Zeta potential (mv)	Entrapment efficiency
SP1	549.2	-30.2	91.32
SP2	561.7	-29.6	90.42
SP3	562.7	-30.8	93.71
SP4	559.3	-30.6	91.37
SP5	561.8	-29.9	90.78

Table 5. Growth inhibition of phytosomal batch of SP2 by using the Trypan blue dye exclusion assay.

S.N.	Sample	Concentration ($\mu\text{g/ml}$)	Absorbance(nm)	%inhibition	STK10
1.	Control	-	3.023		
2.	Std. Dihydropyrimidine Dehydrogenase	10	0.946	68.71	63.74
		30	0.734	75.72	
		80	0.687	77.28	
3.	Optimized batch (SP2)	10	2.376	21.41	87.56
		30	1.990	34.18	
		80	0.740	74.53	

Viable cells do not take on colour, while dead cells take on colour [15, 16]. However, if they are kept longer, the living cells create and take on colour (Figure 8). Growth inhibition rate is calculated using the following formula:

$$\text{Growth inhibition(\%)} = 100 - \frac{\text{Total cells} - \text{death cells}}{\text{Total cells}} \times 100$$

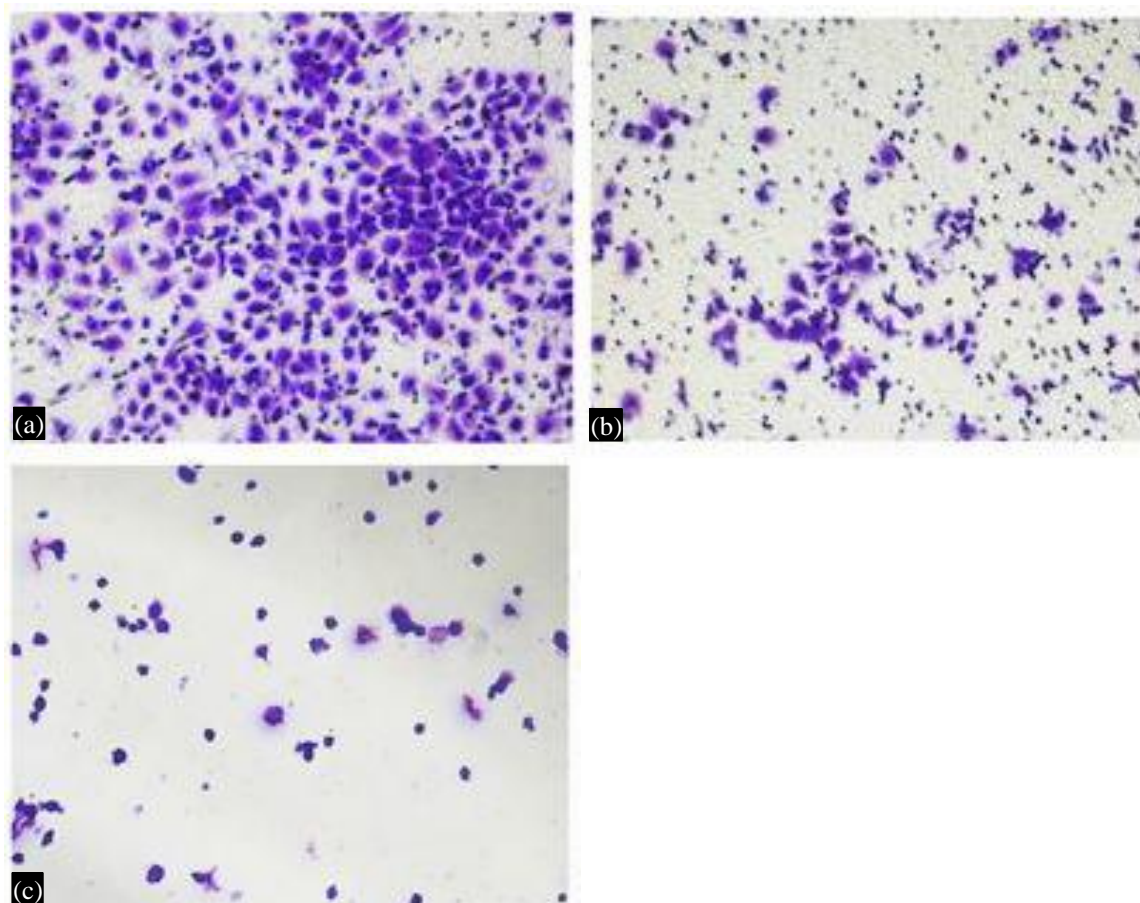


Figure 8. Schematic diagram of Trypan blue dye exclusion assay. (a) Control; (b) Standard; (c) Sample

CONCLUSION

The Phytosomal formulations are found to be in appropriate range (in nm) which could show sustained and controlled release in cancer cells as compared to systemic circulation. Although more research is needed for clinical applications, there are opportunities for prospective treatment options by enhancing nanotechnology. In conclusion, the developed Spirulina extract-loaded Phytosomes, specifically the optimized batch SP2, demonstrated promising characteristics. The formulation exhibited favorable particle size, zeta potential, and entrapment efficiency, indicating its potential for enhanced drug delivery. The In-Vitro drug release study revealed effective controlled release patterns, with SP2 displaying a notable release percentage of 90.87% at the 12th hour. Stability studies confirmed the robustness of the formulation over a two-month period, with minimal changes in particle size, zeta potential, and drug entrapment. The Trypan blue dye exclusion assay further demonstrated the growth inhibition potential of SP2, reinforcing its potential anticancer activity. Overall, the developed Spirulina Phytosomes present a promising approach for delivering the therapeutic benefits of Spirulina, addressing issues of bioavailability and providing a foundation for further exploration of its anticancer properties. This research contributes valuable insights into the development of herbal formulations with improved pharmacokinetic properties for potential anticancer applications.

Declaration of Interest

There is no conflict of interest.

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