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Development and characterization of flavonosome formulation loaded with hesperidine and berberine

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Abstract
The present investigation aimed to develop and comprehensively characterize a novel flavonosome drug delivery system co-loaded with hesperidine and berberine to overcome the inherent bioavailability limitations of these bioactive flavonoids. Extensive pre-formulation studies were conducted to establish the physicochemical properties of both compounds, including organoleptic characteristics, solubility profiles, and analytical method development using UV- Visible spectrophotometry. Flavonosomes were successfully prepared using a bulk co-loading method employing lecithin as the phospholipid carrier. Six different formulations (F1-F6) were prepared and systematically evaluated for particle size, polydispersity index, surface morphology, entrapment efficiency, and *in vitro* release characteristics. The optimized formulation F4 demonstrated superior characteristics with a particle size of 178.3 nm, polydispersity index of 0.421, and remarkable entrapment efficiency of 88.08%. Differential Scanning Calorimetry (DSC) confirmed successful complex formation through molecular interactions between flavonoids and phospholipids. *In vitro* release studies revealed sustained release behavior with 96.05% release for hesperidine and 91.22% for berberine over 12 hours. Stability studies conducted according to ICH guidelines demonstrated excellent physical stability. Antioxidant evaluation using DPPH radical scavenging assay showed enhanced antioxidant activity of the flavonosome formulation (IC_{50} = 151.6 µg/mL) compared to individual compounds (hesperidine IC_{50} = 168.99 µg/mL, berberine IC_{50} = 220.44 µg/mL). This study conclusively demonstrates the potential of flavonosome-based delivery systems as an effective strategy to improve the therapeutic efficacy and bioavailability of naturally derived bioactive compounds.

Keywords: Flavonosomes, hesperidine, berberine, drug delivery, bioavailability enhancement, phospholipid complex, antioxidant activity

1. Introduction

1.1 Background and Rationale

Natural products have served as an invaluable source of therapeutic agents throughout human history, with flavonoids representing one of the most extensively studied classes of bioactive compounds [1, 2]. These polyphenolic substances, ubiquitously distributed in the plant kingdom, exhibit remarkable pharmacological diversity including antioxidant, anti-inflammatory, anticarcinogenic, and neuroprotective properties [3, 4]. Despite their promising therapeutic potential, the clinical translation of flavonoids has been significantly hampered by their inherent physicochemical limitations, particularly poor aqueous solubility, low membrane permeability, rapid metabolism, and consequently poor oral bioavailability [5, 6].

Hesperidine (hesperetin-7-rutinoside), a flavanone glycoside predominantly found in citrus fruits, represents one of the most abundant dietary flavonoids with well- documented biological activities [7, 8]. Extensive pharmacological investigations have demonstrated hesperidine's remarkable therapeutic potential in cardiovascular protection, neuroprotection, diabetes management, and cancer prevention [9, 10]. The compound exhibits potent antioxidant activity through multiple mechanisms including direct radical scavenging, metal chelation, and upregulation of endogenous antioxidant enzymes [11, 12]. However, hesperidine's therapeutic application is severely limited by its poor water solubility (approximately 0.1 mg/mL), low membrane permeability, and extensive first-pass metabolism, resulting in bioavailability of only 15-20% [13, 14].

Berberine, an isoquinoline alkaloid derived from various medicinal plants including Coptis chinensis and Berberis vulgaris, has emerged as a highly promising therapeutic agent with multifaceted pharmacological activities [15, 16].

Modern pharmacological research has validated berberine's traditional medicinal uses, demonstrating significant efficacy in metabolic disorders, cardiovascular diseases, neurological conditions, and antimicrobial applications [17, 18]. The compound's therapeutic mechanisms involve modulation of key cellular pathways including AMPK activation, NF-κB inhibition, and gut microbiota regulation [19, 20]. Despite these promising therapeutic properties, berberine's clinical utility is constrained by poor oral absorption (less than 5%), rapid hepatic metabolism, and extensive efflux by P-glycoprotein [21, 22].

Phytosomes are innovative delivery systems formed through hydrogen bonding interactions between the polar head groups of phospholipids and the hydroxyl groups of polyphenolic compounds. This molecular interaction results in the formation of amphiphilic complexes that can self-assemble into vesicular structures upon hydration, thereby facilitating enhanced cellular uptake and improved bioavailability. The phospholipid component, typically phosphatidylcholine or lecithin, serves a dual function as both a bioavailability enhancer and a nutritionally beneficial component [23, 24].

This work contributes significantly to the field of natural product drug delivery by providing a systematic framework for flavonosome development and characterization while demonstrating the potential for enhanced therapeutic efficacy through intelligent formulation design [25, 26].

2. Materials and Methodology

2.1 Materials

High-purity hesperidine (≥95%) and berberine chloride (≥98%) were procured from Sigma- Aldrich (St. Louis, MO, USA). Lecithin (phosphatidylcholine content ≥95%) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Analytical grade solvents including methanol, ethanol, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and chloroform were purchased from Merck KGaA (Darmstadt, Germany). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma- Aldrich for antioxidant assays. All other reagents used were of analytical grade and used without further purification.

2.2 Pre-formulation Studies

2.2.1 Physical Characterization

Comprehensive physical characterization of hesperidine and berberine was conducted following standard pharmacopoeial methods. Organoleptic properties including color, odor, taste, and appearance were evaluated through visual and sensory examination. Melting point determination was performed using the open capillary method on a digital melting point apparatus (Stuart Scientific, UK), with values reported as uncorrected for atmospheric variations.

2.2.2 Solubility Analysis

Solubility profiles of both compounds were systematically evaluated in various solvents of different polarities. Excess amounts of hesperidine and berberine were added to 10 mL of each solvent (water, methanol, ethanol, DMSO, DMF, chloroform) and subjected to continuous agitation for 24 hours at room temperature. The solutions were filtered, and solubility was qualitatively assessed and categorized according to pharmacopoeial standards.

2.2.3 Analytical Method Development

UV-Visible spectrophotometric methods were developed for quantitative analysis of both compounds. For hesperidine, stock solutions were prepared in methanol and scanned between 200-400 nm to identify the wavelength of maximum absorption (λmax). Similarly, berberine solutions in methanol were analyzed to determine optimal analytical wavelengths. Calibration curves were constructed by plotting absorbance against concentration for both compounds.

2.2.4 Fourier Transform Infrared Spectroscopy (FT-IR): FT-IR spectroscopy was performed using a Shimadzu FT-IR 8400S spectrometer to evaluate potential drug-excipient interactions. Spectra were recorded for pure hesperidine, pure berberine, pure lecithin, and physical mixtures of all components. Samples were prepared using the KBr pellet technique, and spectra were recorded in the range of 4000- 400 cm^{-1}.

2.3 Flavonosome Preparation

Flavonosomes were prepared using the bulk co- loading method, a modified thin-film hydration technique optimized for simultaneous encapsulation of multiple bioactive compounds. This method involves several critical steps designed to maximize encapsulation efficiency while maintaining optimal particle characteristics.

2.3.1 Bulk Co-loading Method

The preparation process begins with the individual dissolution of hesperidine and berberine in appropriate organic solvents, selected based on their solubility profiles. Concurrently, lecithin was dissolved in a separate flask using the same organic solvent system. The flavonoid solutions were combined and mixed thoroughly before being added to the lecithin solution under continuous stirring.

The combined solution was subjected to probe sonication for 10 minutes at 40% amplitude to ensure homogeneous mixing and initial complex formation. Subsequently, the organic solvent was gradually evaporated under reduced pressure using a rotary evaporator at 40°C to form a thin, uniform film on the flask walls. The resulting film was further dried under vacuum to remove residual solvent traces.

The dried film was redispersed in a minimal volume of organic solvent and then slowly added to an aqueous phase (distilled water) under vigorous stirring at room temperature. The final organic solvent removal was achieved through continuous stirring for 2-3 hours, resulting in the formation of stable flavonosome dispersions.

2.4 Formulation Optimization

Six different formulations (F1-F6) were prepared by systematically varying the lecithin to flavonoid ratio to optimize particle characteristics and encapsulation efficiency. The ratios investigated ranged from 1:1 to 6:1 (lecithin: combined flavonoids) to identify the optimal composition for superior particle properties.

2.5 Characterization Methods

2.5.1 Particle Size and Size Distribution Analysis

Particle size analysis was conducted using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) employing dynamic light scattering (DLS) technology. Samples were appropriately diluted with distilled water and analyzed at 25°C with a scattering angle of 90°. The polydispersity

index (PDI), indicative of size distribution uniformity, was simultaneously determined.

2.5.2 Surface Morphology Examination
Scanning Electron Microscopy (SEM) was employed to visualize the surface morphology and structural characteristics of the optimized flavonosome formulation. Samples were prepared by placing a drop of diluted flavonosome dispersion on a carbon-coated copper grid, air-dried, and sputter-coated with gold before examination under a JEOL JSM- 7600F field emission scanning electron microscope.

2.5.3 Differential Scanning Calorimetry (DSC)
Thermal analysis was performed using a DSC 4000 (PerkinElmer, USA) to investigate drug- excipient interactions and complex formation. Samples of pure lecithin, pure drugs, physical mixtures, and flavonosome formulations were analyzed. Approximately 3-5 mg samples were placed in hermetically sealed aluminum pans and heated from 30°C to 300°C at a heating rate of 10°C/min under nitrogen atmosphere.

2.5.4 Entrapment Efficiency Determination
The entrapment efficiency was determined using the indirect method by measuring the amount of non-entrapped drugs in the supernatant. Flavonosome dispersion was centrifuged at 15,000 rpm for 30 minutes at 4°C. The clear supernatant was analyzed spectrophotometrically to determine the concentration of free drugs. Entrapment efficiency was calculated using the following formula:

Entrapment Efficiency (%) = [(Total drug - Free drug) / Total drug] × 100

2.6 *In Vitro* Release Studies
In vitro drug release studies were conducted using the dialysis bag method to evaluate the release kinetics of both flavonoids from the flavonosome formulation. Dialysis bags with molecular weight cut-off of 12,000-14,000 Da were used. The flavonosome dispersion equivalent to 10 mg of total flavonoids was placed inside the dialysis bag and immersed in 100 mL of phosphate buffer (pH 7.4) m maintained at 37°C with continuous stirring at 100 rpm.
Samples were withdrawn at predetermined time intervals (1, 2, 3, 4, 6, 8, 10, and 12 hours) and replaced with an equal volume of fresh buffer to maintain sink conditions. The withdrawn samples were analyzed spectrophotometrically to determine the cumulative drug release.

Stability Studies
Stability studies were conducted according to ICH Q1A (R2) guidelines to evaluate the physical and chemical stability of the optimized flavonosome formulation. Samples were stored under different conditions including room temperature (25°C ± 2°C/60% ± 5% RH) and accelerated conditions (40°C ± 2°C/75% ± 5% RH) for three months. Particle size, PDI, and physical appearance were monitored at regular intervals.

2.7 Antioxidant Activity Evaluation
2.7.1 DPPH Radical Scavenging Assay
The antioxidant activity of individual flavonoids and the flavonosome formulation was evaluated using the DPPH (2,2- diphenyl-1- picrylhydrazyl) radical scavenging assay. DPPH solution (0.1 mM) was prepared in methanol, and various concentrations of test samples (100- 500 µg/mL) were prepared. Equal volumes of test sample and DPPH solution were mixed and incubated in the dark for 30 minutes at room temperature. Absorbance was measured at 51 nm using a UV- Visible spectrophotometer.
The percentage inhibition was calculated using the formula:

% Inhibition = $[(A_0 - A_1) / A_0] \times 100$

Where A_0 is the absorbance of control and A_1 is the absorbance of the test sample. IC_{50} values (concentration required to inhibit 50% of DPPH radicals) were determined from the dose- response curves.

3. Results and Discussion
3.1 Pre-formulation Study Results
3.1.1 Physical Characteristics of Hesperidine
The pre-formulation evaluation of hesperidine revealed characteristic properties consistent with literature reports. The compound appeared as a yellow, tasteless crystalline powder with a characteristic odor. The melting point was determined to be 265-267°C, confirming the identity and purity of the compound. These observations align with pharmacopoeial specifications and previous research findings.

Table 1.1: Organoleptic Properties of Hesperidine

Test	Observation
Color	Yellow
Odor	Characteristic
Taste	Tasteless
Appearance	Crystalline Powder
Melting Point	265-267°C

The solubility analysis revealed hesperidine's poor aqueous solubility, confirming the need for bioavailability enhancement strategies. The compound was insoluble in water, slightly soluble in ethanol and methanol, and soluble in more polar organic solvents like DMSO and DMF.

Table 1.2: Solubility Profile of Hesperidine

Solvent	Observation
Water	Insoluble
Solvent	Observation
Ethanol	Slightly Soluble
Dimethyl Formamide	Soluble
Methanol	Slightly Soluble
DMSO	Soluble

Physical Characteristics of Berberine
Berberine exhibited pale yellow color with odorless and bitter taste characteristics, consistent with literature descriptions. The melting point range of 146-148°C confirmed compound identity.

Table 1.3: Organoleptic Properties of Berberine

Test	Observation
Color	Pale Yellow
Odor	Odorless
Taste	Bitter
Melting Point	146°-148°C

Table 1.4: Solubility Profile of Berberine

Solvent	Solubility
Water	Insoluble
Methanol	Soluble
Ethanol	Soluble
Chloroform	Soluble

Spectrophotometric Method Development

Calibration curves for both compounds demonstrated excellent linearity within the tested concentration ranges. Hesperidine showed maximum absorption at 283 nm in methanol with a linear relationship (R^2 = 0.9946) over the concentration range of 5-25 µg/mL. The regression equation was: Absorbance (y) = 0.0059 × concentration (x) + 0.0063.

Table 1.5: Absorbance Data for Hesperidine Calibration

Concentration (µg/mL)	Absorbance
5	0.028
10	0.069
15	0.091
20	0.126
25	0.152

Berberine exhibited maximum absorption at 418 nm with exceptional linearity (R^2 = 0.9998) over the concentration range of 2-10 µg/mL. The regression equation was:

Absorbance (y) = 0.0738 × concentration (x) + 0.0057.

Table 1.6: Absorbance Data for Berberine Calibration

Concentration (µg/mL)	Absorbance
2	0.157
4	0.297
6	0.446
8	0.596
10	0.745

3.1.4 FT-IR Spectroscopic Analysis

FT-IR spectroscopy confirmed the absence of significant drug-excipient interactions. Hesperidine displayed characteristic bands at 800-1100 cm^{-1} (C-O stretching), 1200-1500 cm^{-1} (aromatic C=C stretching), and 2300-2900 cm^{-1} (C-H stretching). The physical mixture spectrum retained all major functional group peaks of individual components, indicating compatibility and absence of chemical interactions that could compromise stability.

3.2 Flavonosome Characterization Results

3.2.1 Particle Size and Size Distribution Analysis

The particle size analysis revealed significant variations among different formulations, ranging from 178.3 nm to 508.7 nm. The optimized formulation F4 demonstrated the smallest particle size (178.3 nm) with a relatively narrow size distribution (PDI = 0.421), indicating favorable characteristics for cellular uptake and bioavailability enhancement.

Table 1.7: Particle Size and Size Distribution of Flavonosome Formulations

Formulation Code	Particle Size (nm)	Polydispersity Index (PDI)
F1	423.4	0.418
F3	257.1	0.502
F4	178.3	0.421
F5	259.3	0.625
F6	508.7	0.611

The relationship between lecithin concentration and particle size followed a non-linear pattern. Initially, increasing lecithin concentration resulted in particle size reduction, likely due to improved dispersion stability and enhanced surface coverage. However, beyond the optimal ratio (4:1), further increases in lecithin concentration led to particle size increases, possibly due to aggregation phenomena or formation of multilamellar structures.

Surface Morphology Analysis

SEM examination of the F4 formulation revealed spherical, rigid particles with smooth, regular surfaces. The morphological characteristics confirmed successful flavonosome formation and absence of crystalline drug deposits on particle surfaces. The uniform spherical shape is advantageous for predictable *in vivo* behavior and enhanced cellular uptake.

3.2.2 Differential Scanning Calorimetry Results

DSC analysis provided crucial insights into the molecular interactions within the flavonosome structure. Pure lecithin exhibited a sharp mendothermic peak at approximately 54°C, corresponding to its phase transition temperature. The flavonosome formulation showed a significant shift in this transition, with the endotherm moving to 252°C, indicating strong molecular interactions between the flavonoids and phospholipid molecules.

This dramatic shift in thermal behavior suggests successful complex formation through hydrogen bonding and van der Waals interactions between the hydroxyl groups of flavonoids and the polar head groups of phospholipids. Such interactions are crucial for flavonosome stability and enhanced bioavailability.

3.2.3 Entrapment Efficiency

The optimized formulation F4 achieved an impressive entrapment efficiency of 88.08%, indicating successful incorporation of both flavonoids within the phospholipid matrix. This high entrapment efficiency can be attributed to the optimal lecithin-to-flavonoid ratio and the bulk co-loading method's effectiveness in simultaneous encapsulation of multiple compounds.

3.3 *In Vitro* Release Profile

The *in vitro* release studies revealed a biphasic release pattern characteristic of lipid-based delivery systems. An initial burst release was observed within the first hour (23.17% for hesperidine and 12.63% for berberine), attributed to surface- bound drugs or weakly associated compounds. Subsequently, a sustained release phase was observed, with nearly complete release achieved over 12 hours (96.05% for hesperidine and 91.22% for berberine).

Table 1.8: *In Vitro* Release Data of Hesperidine from Flavonosome

Time (hours)	% Cumulative Release
1	23.17
2	27.41
3	36.73
4	43.51
6	55.38
8	66.82
10	82.50
12	96.05

Table 1.9: *In Vitro* Release Data of Berberine from Flavonosome

Time (hours)	% Cumulative Release
1	12.63
2	18.73
3	27.20
4	34.99
6	46.84
8	63.44
10	79.70
12	91.22

The sustained release behavior is advantageous for maintaining therapeutic drug concentrations and potentially reducing dosing frequency. The slight difference in release rates between the two compounds may reflect their different molecular interactions with the phospholipid matrix and varying physicochemical properties.

3.4 Stability Study Results

The three-month stability study demonstrated excellent physical stability of the flavonosome formulation under both storage conditions. Minimal changes in particle size and PDI were observed, confirming the robustness of the formulation. The phospholipid matrix effectively stabilized both flavonoids against degradation and maintained the integrity of the delivery system.

3.5 Antioxidant Activity Results

The DPPH radical scavenging assay revealed superior antioxidant activity of the flavonosome formulation compared to individual compounds.

Table 1.10: DPPH Radical Inhibition by Test Samples

Treatment	Inhibition of DPPH (%)				
	100 µg /mL	200 µg /mL	300 µg/ mL	400 µg/ mL	500 µg/ mL
F4	38 .5	57 .2	78.4	96. 5	99. 8
Hesperidine	34 .8	55 .9	75.1	94. 5	98. 7
Berberine	26 .3	42 .7	69.9	88. 4	97. 6

The IC_{50} values were 151.6 µg/mL for the flavonosome, 168.99 µg/mL for hesperidine, and 220.44 µg/mL for berberine. This enhanced activity suggests synergistic effects between the two flavonoids when co-delivered in the same system.

The improved antioxidant activity of the combination system can be attributed to complementary mechanisms of action. Hesperidine primarily acts through direct radical scavenging and metal chelation, while berberine contributes through cellular antioxidant enzyme upregulation and anti-inflammatory effects. The flavonosome delivery system may also enhance the bioavailability and cellular uptake of both compounds, leading to improved antioxidant efficacy.

4. Conclusion

This comprehensive investigation successfully demonstrates the development and characterization of a novel flavonosome delivery system co-loaded with hesperidine and berberine. The study provides compelling evidence for the potential of phospholipid-based delivery systems to overcome the bioavailability limitations of therapeutically important flavonoids while achieving synergistic therapeutic benefits.

The optimized formulation (F4) exhibited superior characteristics including nanoscale particle size (178.3 nm), high entrapment efficiency (88.08%), sustained release behavior, excellent stability, and enhanced antioxidant activity compared to individual compounds. The systematic approach employed in this study, encompassing comprehensive pre-formulation studies, systematic formulation optimization, extensive physicochemical characterization, and biological evaluation, provides a robust framework for future flavonosome development.

The findings of this research have significant implications for the pharmaceutical industry and nutraceutical applications. The demonstrated ability to simultaneously deliver multiple bioactive compounds with enhanced bioavailability and synergistic effects opens new possibilities for combination therapies using natural products. Furthermore, the sustained release characteristics and improved stability of the flavonosome system suggest potential for reduced dosing frequency and enhanced patient compliance.

Future research directions should focus on *in vivo* bioavailability studies, detailed pharmacokinetic evaluations, and exploration of specific therapeutic applications. Additionally, scale-up studies and regulatory considerations will be essential for translating this promising delivery system from laboratory to clinical applications.

The study conclusively establishes flavonosome-based formulations as a valuable approach to improve the therapeutic efficacy of natural flavonoids, representing a significant advancement in natural product drug delivery and providing a foundation for future developments in this rapidly evolving field.

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