Development and In Vitro Evaluation of Resveratrol–Betulin Co-Loaded Liposomes for Enhanced Anti-Inflammatory and Antioxidant Activity

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Abstract

Background: Resveratrol and betulin are poorly water-soluble bioactive compounds investigated for antioxidant and anti-inflammatory activity. Their limited aqueous solubility presents a formulation challenge. Objective: This study evaluated phosphatidylcholine/cholesterol liposomes for co-delivery of resveratrol and betulin and compared three lipid levels while keeping the total drug amount constant. Methods: Co-loaded liposomes (F1–F3) were prepared by ethanol injection using a phosphatidylcholine: cholesterol mass ratio of 2:1. The formulations were evaluated for particle size, polydispersity index (PDI), zeta potential, morphology, entrapment efficiency, in vitro drug release, COX-2 inhibition and DPPH radical-scavenging activity. Results: F2, containing 200 mg phosphatidylcholine and 100 mg cholesterol, showed the smallest particle size (142.7 ± 2.8 nm), lowest PDI (0.256 ± 0.02) and zeta potential of −28.3 ± 1.0 mV. Entrapment efficiencies were 81.7 ± 2.1% for resveratrol and 85.4 ± 2.3% for betulin. At 24 h, cumulative release from F2 was 91.8 ± 2.3% for resveratrol and 89.2 ± 2.2% for betulin. At 100 µg/mL, F2 produced 91.6 ± 2.5% COX-2 inhibition and 92.4 ± 2.6% DPPH scavenging, compared with 71.2 ± 2.0% and 76.5 ± 2.2%, respectively, for the free-drug comparator. Conclusion: F2 showed the most favourable overall physicochemical and in vitro biochemical performance among the tested formulations. The findings support further investigation of resveratrol–betulin co-loaded liposomes; however, the supplied data do not establish pharmacological synergy, in vivo efficacy, pharmacokinetic benefit or long-term stability.

Keywords: resveratrol; betulin; liposomes; ethanol injection; phosphatidylcholine; cholesterol; COX-2; DPPH; nanocarrier; drug delivery

1. Introduction

Inflammation is a protective biological response involving coordinated vascular, cellular and molecular events following tissue injury or infection. When excessive or persistent, inflammatory signalling can contribute to tissue damage and chronic disease. Conventional anti-inflammatory medicines, including nonsteroidal anti-inflammatory drugs and corticosteroids, remain clinically important, but their use may be limited by adverse effects and patient-specific safety considerations. This has encouraged continued investigation of bioactive natural products and delivery systems that can improve pharmaceutical performance.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a stilbene polyphenol investigated for antioxidant and anti-inflammatory effects. Reported mechanisms include modulation of inflammatory signalling pathways, while its low aqueous solubility and extensive metabolism remain important formulation barriers. Liposomal and other

lipid-based systems have therefore been investigated as approaches to improve its formulation performance [1,2,7].Betulin is a lupane-type pentacyclic triterpenoid chemically identified as lup-20(29)-ene-3β,28-diol. It contains two hydroxyl groups and is structurally distinct from betulinic acid. Its high lipophilicity and limited aqueous solubility make lipid-based delivery a rational formulation strategy for exploratory development [3,4,12].Liposomes are phospholipid vesicles that can incorporate hydrophobic molecules within their lipid bilayer. Particle size, membrane composition, surface charge and preparation conditions influence drug loading, release and colloidal behaviour. Ethanol injection is a relatively simple preparation approach in which an ethanolic lipid phase is introduced into an aqueous phase, allowing solvent diffusion and vesicle formation [5,6].The present study was designed to develop resveratrol–betulin co-loaded liposomes using phosphatidylcholine and cholesterol and to evaluate the effect of lipid level on particle size, PDI, zeta potential, morphology, entrapment efficiency, in vitro drug release, COX-2 inhibition and DPPH radical-scavenging activity.

2. Rationale, Research Gap and Objective

Published literature supports the formulation interest of poorly water-soluble natural compounds such as resveratrol and betulin and the use of liposomes for hydrophobic drug delivery. The specific co-loaded resveratrol–betulin liposomal system evaluated here requires experimental assessment rather than an assumption of pharmacological synergy. The formulation series varied total lipid while maintaining a constant total drug amount. The primary objective was to identify the formulation with the most favourable balance of particle size, PDI, zeta potential, entrapment efficiency, release behaviour and in vitro biological activity.

3. Materials and Methods

3.1 Materials

Resveratrol and betulin were used as active compounds. Phosphatidylcholine and cholesterol were used as lipid components. Absolute ethanol was used for the lipid phase and phosphate-buffered saline (PBS, pH 7.4) as the aqueous phase. Methanol and chloroform were used in preformulation solubility studies. Dimethyl sulfoxide (DMSO) was used as the solvent for samples in the COX-2 assay. Diclofenac sodium and ascorbic acid were used as reference standards for the COX-2 and DPPH assays, respectively.

3.2 Preformulation Studies

Organoleptic characteristics were assessed visually. Solubility was evaluated by a shake-flask approach in distilled water, PBS (pH 7.4), methanol, ethanol and chloroform. Absorption maxima were determined by scanning methanolic solutions from 200 to 400 nm. Melting points were measured by the capillary method. Partitioning between n-octanol and water was used to estimate experimental log P. FTIR spectra were recorded over 4000–400 cm^{-1} for the pure compounds and selected physical mixtures.

3.3 Preparation of Co-Loaded Liposomes

Phosphatidylcholine and cholesterol were dissolved in 10 mL absolute ethanol at a constant 2:1 phosphatidylcholine:cholesterol mass ratio. Resveratrol (15 mg) and betulin (15 mg) were incorporated into the ethanolic lipid phase, giving a constant total drug amount of 30 mg per batch. The aqueous phase consisted of 20 mL PBS (pH 7.4) maintained at 40 ± 2°C under stirring at 800 rpm. The ethanolic phase was injected at approximately 1 mL/min. The dispersion was stirred for 1 h at room temperature and then probe-sonicated at 40% amplitude for 5 min using 30 s on/30 s off pulses. The dispersion was filtered through a 0.45 µm membrane and stored in airtight glass vials at 4°C.

The actual drug-to-total-lipid ratios were calculated from the quantities used: 1:7.5 for F1 (30:225 mg), 1:10 for F2 (30:300 mg) and 1:12.5 for F3 (30:375 mg). Thus, 1:10 is specific to F2 and should not be reported as a common ratio for all formulations.

Component	F1	F2	F3	Function
Phosphatidylcholine (mg)	150	200	250	Lipid bilayer former
Cholesterol (mg)	75	100	125	Membrane stabilizer
Resveratrol (mg)	15	15	15	Active compound
Betulin (mg)	15	15	15	Active compound
Ethanol	10 mL	10 mL	10 mL	Organic solvent
PBS pH 7.4	20 mL	20 mL	20 mL	Aqueous phase
Total lipid (mg)	225	300	375	Phosphatidylcholine + cholesterol
Drug:total-lipid (w/w)	1:7.5	1:10	1:12.5	30 mg total drug / total lipid

Table 1. Composition of resveratrol–betulin co-loaded liposomes.

3.4 Liposome Characterization

Particle size, PDI and zeta potential were determined by dynamic light scattering at 25 ± 1°C after 1:10 dilution. Measurements were performed in triplicate. Morphology of F2 was examined by transmission electron microscopy after dilution, deposition on a carbon-coated copper grid, negative staining with 1% phosphotungstic acid and air drying. Entrapment efficiency was determined by centrifugation at 15,000 rpm for 30 min at 4°C; free drug was quantified at 306 nm for resveratrol and 210 nm for betulin.

3.5 In Vitro Drug Release

An accurately measured liposomal dispersion was placed in a pre-treated dialysis bag and immersed in 100 mL PBS (pH 7.4) maintained at 37 ± 0.5°C and stirred at 100 rpm. Samples were withdrawn at 0.5, 1, 2, 4, 6, 8, 12 and 24 h and replaced with an equal volume of fresh pre-warmed PBS. Resveratrol and betulin were quantified at 306 and 210 nm, respectively. Results were reported as mean ± SD (n=3).

3.6 COX-2 Inhibition Assay

F1–F3, the free-drug comparator and diclofenac sodium were tested at 10–100 µg/mL. Samples were incubated with COX-2 enzyme and assay buffer at 37°C for 10 min, followed by addition of arachidonic acid and a further 15 min incubation. After termination of the reaction, absorbance was measured at 560 nm. Percentage inhibition was calculated as: % inhibition = [(Acontrol − Asample) / Acontrol] × 100.

3.7 DPPH Radical-Scavenging Assay

A 0.1 mM DPPH solution in methanol was reacted with F1–F3, the free-drug comparator and ascorbic acid at 10–100 µg/mL. Reaction mixtures were incubated in the dark for 30 min at 25 ± 2°C and read at 517 nm. Radical-scavenging activity was calculated from the reduction in absorbance relative to the control [11].

3.8 Data Handling and Statistical Reporting

Data are presented as mean ± standard deviation (SD) with n=3, as reported in the supplied study. Because the underlying replicate-level raw observations were not supplied, no new inferential p-values, ANOVA, post-hoc tests or other significance statistics were generated. Accordingly, comparisons in this manuscript are descriptive and should not be described as statistically significant. Raw replicate data should be retained and analysed before submission if the target journal requires inferential statistics.

4. Results

4.1 Preformulation Findings

Parameter	Resveratrol	Betulin
Appearance	Off-white to pale yellow crystalline powder	White amorphous powder
Aqueous solubility, distilled water (mg/mL)	0.030 ± 0.002	0.005 ± 0.001
Solubility in PBS pH 7.4 (mg/mL)	0.050 ± 0.003	0.008 ± 0.002
Solubility in methanol (mg/mL)	8.72 ± 0.15	2.45 ± 0.08
Solubility in ethanol (mg/mL)	6.35 ± 0.12	1.98 ± 0.06
Solubility in chloroform (mg/mL)	3.20 ± 0.10	6.85 ± 0.14
λmax (nm)	306	210
Melting point (°C)	253–255	256–258
Experimental log P	0.64	1.26

Table 2. Preformulation findings for resveratrol and betulin.

Both compounds showed markedly lower solubility in water and PBS than in the tested organic solvents. Betulin showed the highest measured solubility in chloroform, whereas resveratrol showed the highest measured solubility in methanol. The experimental log P values were 0.64 for resveratrol and 1.26 for betulin. The analytical wavelengths were 306 nm for resveratrol and 210 nm for betulin; the supplied study reported calibration ranges of 2–10 µg/mL with R^2 values of 0.999 and 0.998, respectively.

4.2 FTIR Compatibility

The supplied FTIR results showed retention of the major drug-associated signals after physical mixing with the selected lipid excipients, without a reported new dominant absorption band. For resveratrol, the observed spectrum was interpreted using phenolic O–H, aromatic C=C, phenolic C–O and trans-olefinic features. For betulin, interpretation was restricted to functional groups consistent with its lupane-triterpene structure, including O–H, aliphatic C–H, alkene-associated and C–O/fingerprint features [3,12].

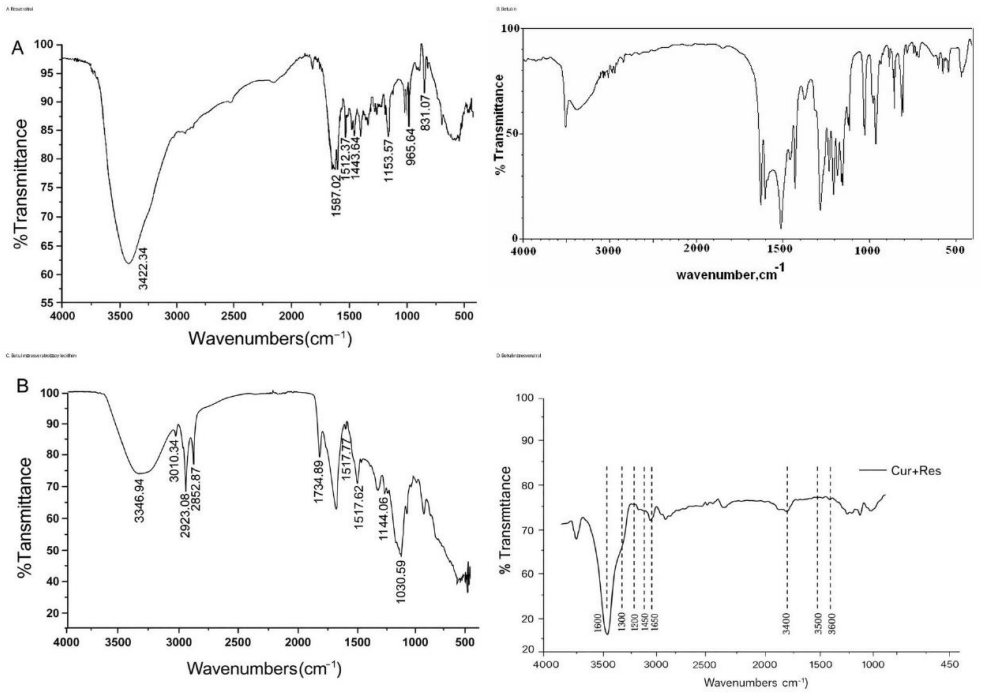

Figure 1. FTIR spectra of resveratrol, betulin and selected physical mixtures

4.3 Liposome Characterization

Formulation	Particle size (nm)	PDI	Zeta potential (mV)
F1	185.4 ± 3.2	0.312 ± 0.01	−21.5 ± 1.2
F2	142.7 ± 2.8	0.256 ± 0.02	−28.3 ± 1.0
F3	168.9 ± 3.5	0.298 ± 0.01	−25.7 ± 1.3

Table 3. Particle size, PDI and zeta potential of the liposomal formulations.

All formulations were nanosized. F2 showed the smallest mean particle size and lowest PDI among the three batches. F2 also showed the most negative zeta potential. These measurements support a comparatively uniform dispersion for F2, while zeta potential alone should not be interpreted as a substitute for a formal long-term stability study.

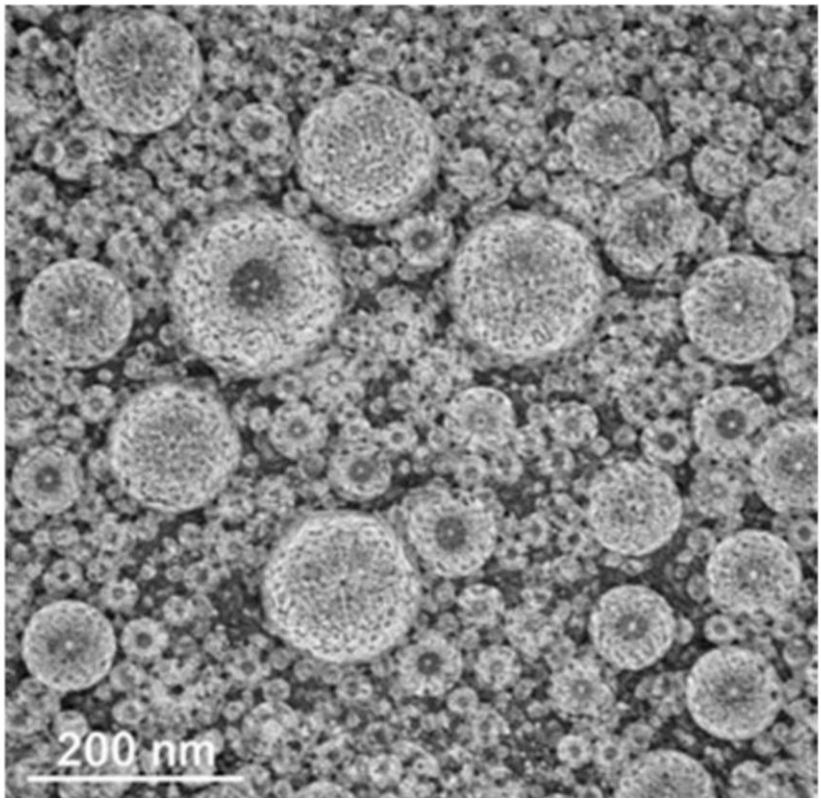

Figure 2. Transmission electron micrograph of optimized liposomal formulation F2; scale bar 200 nm.

4.4 Entrapment Efficiency

Formulation	Resveratrol EE (%)	Betulin EE (%)
F1	68.5 ± 1.8	72.3 ± 2.0
F2	81.7 ± 2.1	85.4 ± 2.3
F3	75.2 ± 1.9	78.6 ± 2.1

Table 4. Entrapment efficiency of resveratrol and betulin in F1–F3.

F2 showed the highest entrapment efficiency for both compounds. Relative to F1, F2 increased the reported entrapment efficiency by 13.2 percentage points for resveratrol and 13.1 percentage points for betulin. F3 showed intermediate values, indicating that increasing total lipid beyond the F2 level did not further improve the measured entrapment under the conditions used. Liposome drug loading is influenced by formulation composition and drug-to-lipid conditions [8,13].

4.5 In Vitro Drug Release

Time (h)	Res-F1	Res-F2	Res-F3	Bet-F1	Bet-F2	Bet-F3
0.5	12.5 ± 0.8	10.2 ± 0.6	11.8 ± 0.7	10.8 ± 0.7	8.9 ± 0.5	10.1 ± 0.6
1	20.3 ± 1.1	17.5 ± 0.9	19.2 ± 1.0	18.6 ± 0.9	15.7 ± 0.8	17.4 ± 0.9
2	32.7 ± 1.3	28.4 ± 1.2	30.8 ± 1.1	29.4 ± 1.2	25.6 ± 1.1	27.8 ± 1.0
4	48.6 ± 1.5	42.3 ± 1.4	45.9 ± 1.3	45.2 ± 1.4	39.8 ± 1.3	42.6 ± 1.2

6	60.4 ± 1.8	55.1 ± 1.6	58.2 ± 1.5	57.9 ± 1.6	52.3 ± 1.5	54.8 ± 1.4
8	70.2 ± 2.0	65.8 ± 1.7	68.9 ± 1.8	68.5 ± 1.9	63.7 ± 1.7	66.2 ± 1.6
12	82.5 ± 2.2	78.6 ± 2.0	80.3 ± 2.1	80.1 ± 2.1	75.4 ± 1.9	77.8 ± 2.0
24	94.2 ± 2.5	91.8 ± 2.3	93.1 ± 2.4	92.6 ± 2.4	89.2 ± 2.2	90.8 ± 2.3

Table 5. Cumulative in vitro release of resveratrol and betulin from F1–F3 (mean ± SD, n=3).

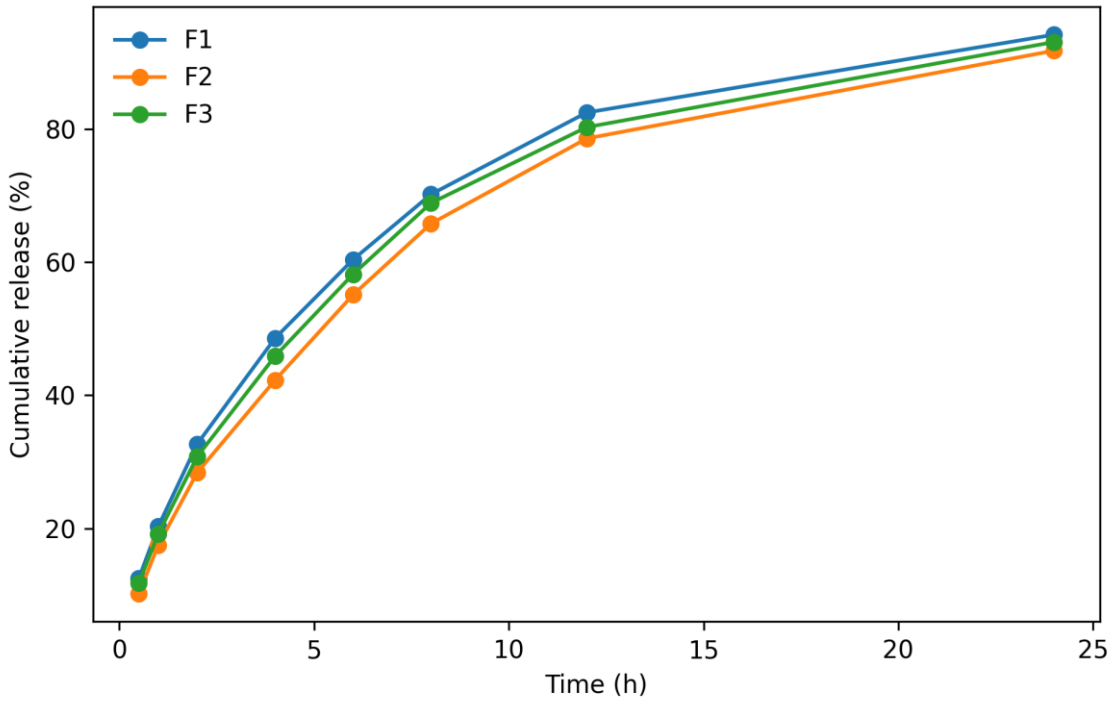

Figure 3. In vitro release profile of resveratrol from liposomal formulations F1–F3.

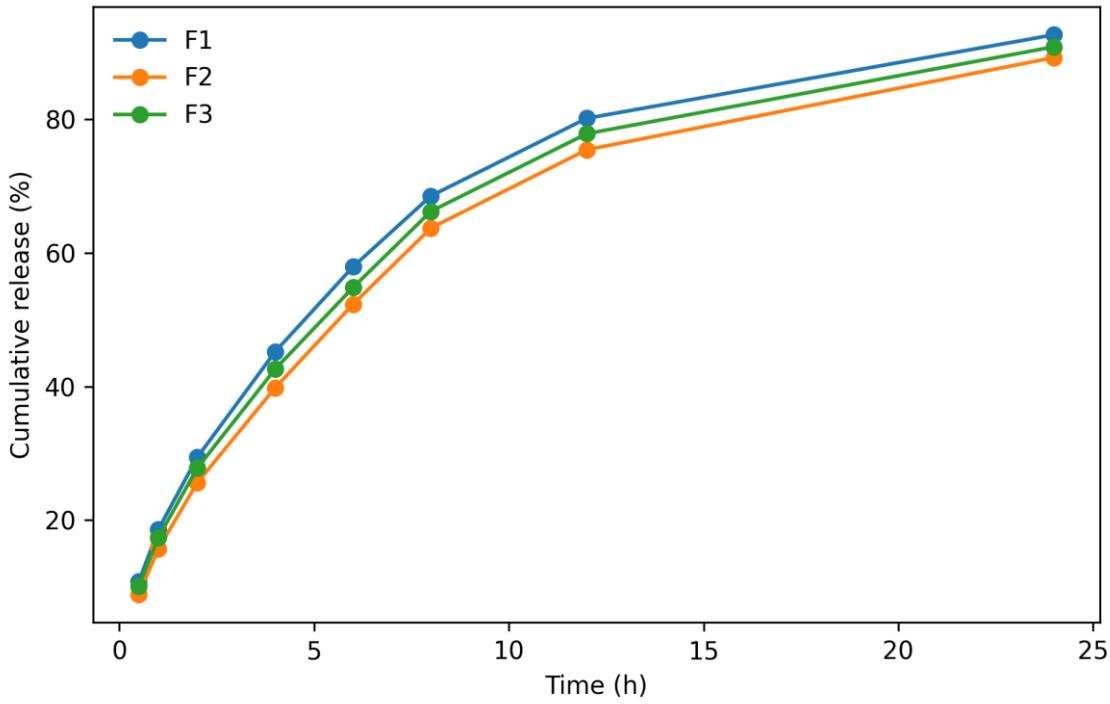

Figure 4. In vitro release profile of betulin from liposomal formulations F1–F3.

All formulations showed an early release phase followed by slower release up to 24 h. F2 consistently showed the lowest cumulative release among the three formulations, reaching 91.8 ± 2.3% for resveratrol and 89.2 ± 2.2% for betulin at 24 h. The profile is compatible with comparatively greater retention within the lipid system, although formal release-kinetic modelling would be required before assigning a mechanistic release model.

4.6 In Vitro COX-2 Inhibition

Concentration (µg/mL)	F1	F2	F3	Free-drug comparator	Diclofenac sodium
10	28.5 ± 1.2	35.2 ± 1.4	31.8 ± 1.3	25.4 ± 1.1	45.6 ± 1.5
25	42.7 ± 1.5	52.6 ± 1.7	47.3 ± 1.6	38.9 ± 1.4	62.8 ± 1.8
50	58.9 ± 1.8	71.4 ± 2.0	64.2 ± 1.9	54.6 ± 1.7	78.5 ± 2.1
75	68.3 ± 2.0	82.7 ± 2.3	74.5 ± 2.1	63.8 ± 1.9	88.2 ± 2.4
100	76.5 ± 2.3	91.6 ± 2.5	83.2 ± 2.2	71.2 ± 2.0	95.4 ± 2.6

Table 6. COX-2 inhibition by liposomal formulations and reference comparators (mean ± SD, n=3).

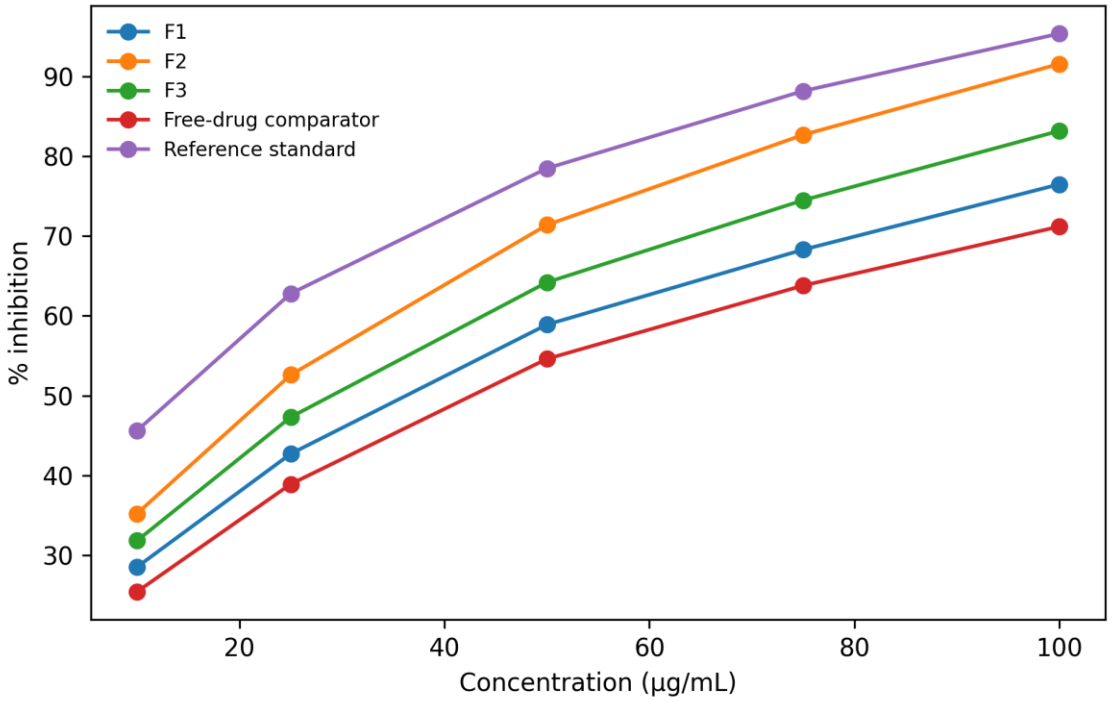

Figure 5. COX-2 inhibition across the tested concentrations.

COX-2 inhibition increased with concentration for all tested samples. F2 showed the highest inhibition among the liposomal formulations at every tested concentration. At 100 µg/mL, F2 produced 91.6 ± 2.5% inhibition compared with 71.2 ± 2.0% for the free-drug comparator, whereas diclofenac sodium produced 95.4 ± 2.6%. Thus, the supplied data show a higher measured enzyme-inhibition response for F2 than for the free-drug comparator, but they do not establish in vivo efficacy, clinical equivalence or pharmacological synergy.

4.7 DPPH Radical-Scavenging Activity

Concentration (µg/mL)	F1	F2	F3	Free-drug comparator	Ascorbic acid
10	32.4 ± 1.3	40.8 ± 1.5	35.6 ± 1.4	28.7 ± 1.2	52.3 ± 1.7
25	46.9 ± 1.6	58.7 ± 1.9	51.2 ± 1.7	42.5 ± 1.5	68.4 ± 2.0
50	61.5 ± 1.9	74.3 ± 2.2	66.8 ± 2.0	57.1 ± 1.8	82.6 ± 2.3
75	72.8 ± 2.1	85.6 ± 2.4	78.4 ± 2.2	68.9 ± 2.0	91.3 ± 2.5
100	80.6 ± 2.3	92.4 ± 2.6	86.9 ± 2.4	76.5 ± 2.2	96.8 ± 2.7

Table 7. DPPH radical-scavenging activity of liposomal formulations and reference comparators (mean ± SD, n=3).

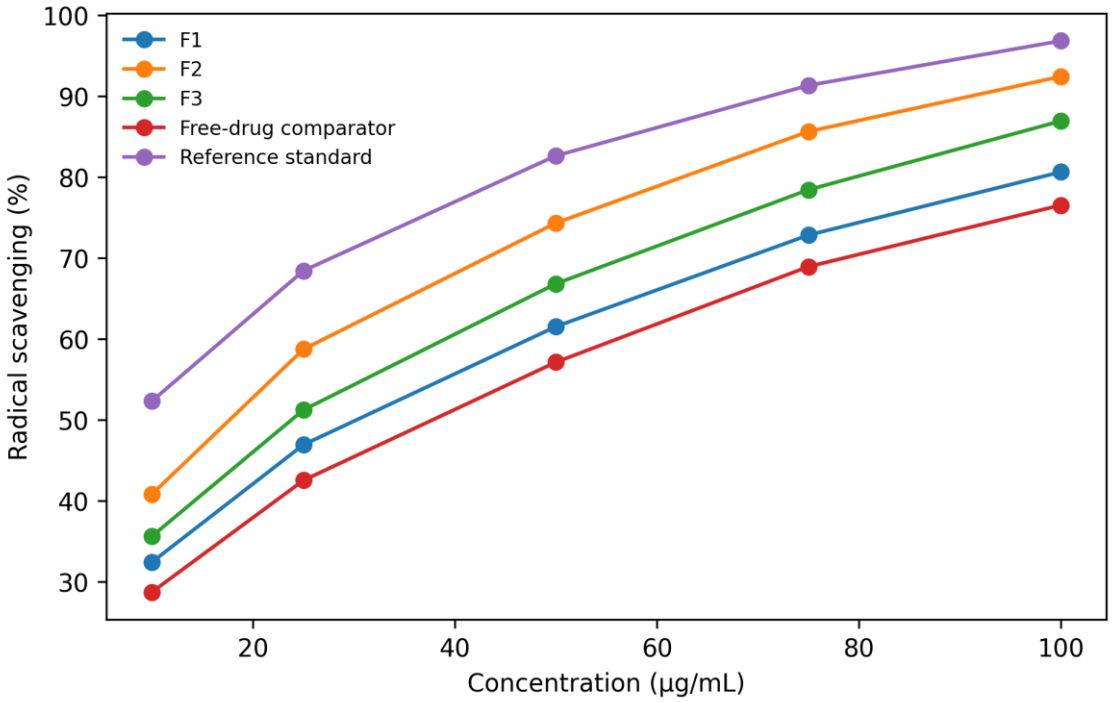

Figure 6. DPPH radical-scavenging activity across the tested concentrations.

DPPH scavenging increased with concentration. F2 showed the highest activity among the liposomal formulations. At 100 µg/mL, F2 showed 92.4 ± 2.6% scavenging compared with 76.5 ± 2.2% for the free-drug comparator, while ascorbic acid showed 96.8 ± 2.7%. These findings indicate improved activity in this chemical assay relative to the free-drug comparator, but DPPH scavenging should not be interpreted as evidence of cellular antioxidant efficacy.

5. Discussion

Both compounds showed low aqueous solubility relative to the organic solvents tested, supporting the formulation rationale for a lipid-based carrier. Betulin showed the higher experimental log P value, consistent with greater lipophilic character under the experimental conditions. These findings are in line with the broader formulation challenge described for resveratrol and betulin [1–4,7].

The ethanol-injection process produced nanosized vesicles across the three formulations. F2 had the smallest particle size and lowest PDI, whereas F3 showed a modest increase in both parameters after further increasing the total lipid level. This non-linear response is plausible because liposome size depends on lipid concentration, membrane packing, cholesterol content, solvent diffusion, mixing and post-formation processing [5,6,10,14]. DLS and zeta potential are comparative physicochemical measurements and are sensitive to sample preparation and measurement conditions.

The formulation composition required an important correction before publication. Because the total drug amount was fixed at 30 mg, the actual drug-to-total-lipid ratios were 1:7.5, 1:10 and 1:12.5 for F1, F2 and F3, respectively. Therefore, the experimental design is best described as lipid-level screening rather than use of a universal 1:10 drug-to-lipid ratio. Drug-to-lipid ratio is a recognized formulation variable that can influence encapsulation and release [8–10].

F2 showed the highest entrapment efficiency for both resveratrol and betulin. The data suggest that the intermediate lipid level used in F2 provided a favourable formulation environment for incorporation of the two hydrophobic compounds. The lower values in F3 indicate that increasing lipid content beyond the F2 level did not further improve measured entrapment under the conditions used. This supports experimental optimization rather than assuming that the highest lipid content is necessarily optimal.

F2 also showed the lowest cumulative release throughout the 24-h study. The release curves displayed an early release phase followed by slower release. Such behaviour can be compatible with release of more accessible or surface-associated drug followed by slower diffusion from the lipid phase; however, a definitive mechanism cannot be assigned without formal kinetic modelling and additional experimental controls.

The biological assays showed the same formulation ranking, with F2 producing the highest measured COX-2 inhibition and DPPH scavenging among F1–F3. At 100 µg/mL, F2 produced 91.6% COX-2 inhibition and 92.4% DPPH scavenging, both higher than the free-drug comparator. Nevertheless, the present dataset does not establish that the two active compounds interact synergistically. Demonstration of pharmacological synergy requires a defined interaction design, such as a combination-index approach, isobologram or response-surface analysis using multiple component ratios. The present study varied lipid level while keeping the drug amounts constant and did not provide such an analysis.

Interpretation should also remain specific to the assay models. DPPH is a chemical radical-scavenging assay and does not reproduce cellular antioxidant defence. A biochemical COX-2 inhibition assay demonstrates enzyme-level activity but cannot establish tissue exposure, pharmacokinetics, toxicity or clinical efficacy. Accordingly, the most defensible interpretation is that F2 showed improved physicochemical and in vitro biochemical performance relative to the supplied free-drug comparator.

6. Conclusion

Resveratrol–betulin co-loaded liposomes were prepared by ethanol injection using phosphatidylcholine and cholesterol. Among the three formulations, F2 containing 200 mg phosphatidylcholine and 100 mg cholesterol showed the most favourable overall performance, with a particle size of 142.7 ± 2.8 nm, PDI of 0.256 ± 0.02, zeta potential of −28.3 ± 1.0 mV and entrapment efficiencies of 81.7 ± 2.1% for resveratrol and 85.4 ± 2.3% for betulin. F2 also showed the lowest cumulative release over 24 h and the highest measured COX-2 inhibition and DPPH radical-scavenging activity among the liposomal formulations. These findings support further investigation of the co-loaded liposomal platform for poorly water-soluble bioactive compounds. However, the present data do not establish pharmacological synergy, cellular efficacy, pharmacokinetic benefit, long-term stability or in vivo therapeutic effectiveness.

7. Declarations

Ethics approval
Not applicable to the reported in vitro physicochemical and biochemical work; no human or animal subjects were reported in the supplied study.

Consent for publication
Not applicable.

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Author contributions
To be completed according to the actual contributions of each author. Authorship should reflect substantive contributions and all listed authors should approve the final manuscript.

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