SUPPLEMENTARY MATERIAL

Double-Loaded Liposomes Encasing Umbelliferone in Hydroxypropyl-β-Cyclodextrin Inclusion Complexes: Formulation, Characterisation and Investigation of Photoprotective Activity

Rucheera R. Verekar [1], Shamshad Bi M. Shaikh [2], Sarita Rebelo [2], Shailendra S. Gurav [1*]

[1] *Department of Pharmacognosy, Goa College of Pharmacy, Goa University, Goa, India*
[2] *School of Biological Sciences and Biotechnology, Goa University, Taleigao Plateau, Taleigao, Goa, India*
(**Corresponding author's email: shailendra.gurav@nic.in*)

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Methodology:

Optimisation of UMB-β-CD molecular inclusion complex

Percent yield

The effectiveness of any particular method of inclusion complex preparation was calculated using the percent yield, which contributes to choose the best production technique. The following formula was used for estimating the practical yield upon weighing the collected inclusion complex [27, 29]:

$$\text{Percent yield} = \frac{Practical\ mass}{Theoretical\ Mass\ (Drug\ +\ Carrier)} x100$$

Assay or Drug Content Estimation

Precisely weighed inclusion complex corresponding to 10 mg of UMB was transferred to a volumetric flask measuring 100 mL and dissolved in a small amount of ethanol. Distilled water was then added to bring the volume up to 100 mL. Following the removal of 1 mL of the solution and its dilution with 10 mL of distilled water, a spectrophotometric measurement was performed at 324.5 nm [25].

Characterisation of optimised UMB-β-CD molecular inclusion complex

Fourier transform infrared spectroscopy (FTIR)

FT-IR analyses were conducted on the plain drug, PL90H, cholesterol, HP-β-CD, physical mixture (PM) and inclusion complex, using an FTIR spectrophotometer (Model: IR Affinity-1S, Shimadzu). A small portion of the test sample was positioned directly beneath the rigidly fixed probe, which was then

scanned in the wave number range of 4000–400 cm^{-1} and analysed for 45 scans. The obtained spectrum was then compared with standard group frequencies of the drug/excipient sample [30, 31].

Saturation solubility studies

The solubility of the optimised inclusion complex (C5) was determined by adding a known excess amount of inclusion complex to 10 mL of dissolution medium (PBS 7.4). The dispersion was held at room temperature for 24 h on the rotary flask shaker; then, the solution was filtered using Whatman filter paper and analysed with a UV-visible spectrophotometer at 324.5 nm [5].

Differential Scanning Calorimetry (DSC) analysis

One of the most popular calorimetric methods for examining the solid-state interaction of a drug with HP-β-CD is DSC. The solid complex and pure drug samples were heated in aluminum pans with flat bottoms between 70°C and 320°C at a constant rate of 10°C per minute by employing alumina as a reference standard in a differential scanning calorimeter [28, 32].

X-ray diffraction (XRD) analysis

In addition to DSC, the powder XRD technique has been widely used to investigate the drug-HP-β-CD interaction. The powder X-ray diffractometer was used to conduct the diffraction experiments. A fixed tube current of 15 mA and a voltage of 40 kV were used to operate the device. The amorphous nature of the inclusion complex was ascertained by scanning the samples at a rate of 10º/min from 3°C to 90°C [33].

Scanning electron microscopy (SEM)

The morphological characteristics of the drug's surface and inclusion complex were examined using a scanning electron microscope. Briefly, the sample was layered on double-sided carbon tape and a brass stub. With the help of the fine auto coater, palladium was coated onto the surface of the powder. Palladium-coated samples were examined using SEM (JEOL model JSM 639OLV) with a digital camera and an increasing voltage of 10 KV. SEM scanning of the sample was carried out using electron beam imaging from the University Science Instrumentation Center (USIC), Goa University, Goa [32].

Ex-vivo skin permeation

Rats' abdomens were shaved with a trimmer. A full-thickness layer of the skin surrounding the abdomen was excised. After removal of the adhered fat and cleaning with isopropyl alcohol to remove any remaining tissue, the skin was washed with PBS (pH 7.4). The skin was placed on the Franz diffusion cell in the proper orientation, with the dermal side confronting the receptor compartment and the stratum corneum side towards the donor compartment [25, 30].

T a b l e S1

Batches of gel formulation

INGREDIENTS	B1	B2	B3	B4
Carbopol 934	1.0%	1.5%	2.0%	2.5%
Drug concentration	1%	1%	1%	1%
Propylene glycol	2.5%	2.5%	2.5%	2.5%
Triethanolamine	q.s	q.s	q.s	q.s
Methyl paraben	0.1%	0.1%	0.1%	0.1%
Propyl paraben	0.01%	0.01%	0.01%	0.01%
Distilled water	q.s	q.s	q.s	q.s

T a b l e S2

Study groups for photoprotective activity

Code	Groups used	No. of animals
G1	Control	06
G2	Placebo UV irradiated group	06
G3	UMB 1 gel (Immediate UV treatment) 0.1% drug	06
G4	UMB 2 gel (UV treatment after 4 h) 0.1% drug	06
G5	DL-UMB-CDLP 1 gel (Immediate UV treatment)	06
G6	DL-UMB-CDLP 2 gel (UV treatment after 4 h)	06

T a b l e S3

% practical yield and % drug content of UMB inclusion complexes

FORMULATION CODE	RATIO	PERCENT YIELD	Drug content (%)
C1	1:1	93.33% ±0.45	97.70% ±0.19
C2	1:2	94.95% ±0.89	93.13% ±0.23
C3	1:3	95.19% ±0.67	95.03% ±0.21
C4	1:1	84.19% ±0.32	82.19% ±0.19
C5	1:2	95.65% ±0.58	98.34% ±0.40
C6	1:3	93.16% ±0.54	96.43% ±0.41
C7	1:1	87.43% ±0.41	83.47% ±0.5
C8	1:2	88.96% ±0.56	93.13% ±0.50
C9	1:3	87.03% ±0.12	94.65% ±0.42
C10	1:1	86.67% ±0.14	77.37% ±0.64
C11	1:2	94.80% ±0.16	89.69% ±0.31
C12	1:3	89.77% ±0.17	93.38% ±0.53

Data are expressed as mean ±SD (n=3)

T a b l e S4

% Drug release of profiles of UMB inclusion complexes

Time (min)	1	3	5	7
UMB	17.09% ±0.24	19.17% ±0.11	20.63% ±0.42	22.10% ±0.6
Physical mixture				
C1	76.18% ±0.20	90 .91% ±0.39	92.45% ±0.3	97.81% ±0.51
C2	82.53% ±0.23	90.84% ±0.13	94.44% ±0.33	97.97% ±0.12
C3	84.55% ±0.38	88.5% ±0.49	90.88% ±0.03	94.0% ±0.26
Kneading method				
C4	55.85% ±0.26	72.36% ±0.11	79.99% ±0.31	87.76% ±0.15
C5	66.65% ±0.05	82.56% ± 0.13	92.27% ±0.15	98.90% ±0.13
C6	61.56% ±0.37	75.52% ±0.14	83.89% ±0.45	89.80% ±0.32
Solvent evaporation method				
C7	8.83% ±0.41	10.19% ±0.28	12.83% ±0.21	16.14% ±0.43
C8	28.53% ±0.11	45.97% ±0.13	58.49% ±0.12	67.97% ±0.10
C9	27.26% ±0.16	42.14% ±0.13	53.99% ±0.15	62.15% ±0.11
Co-evaporation method				
C10	6.29% ±0.13	10.17% ±0.13	12.81% ±0.12	17.38% ±0.25
C11	34.24% ±0.33	54.28% ±0.26	63.08% ±0.21	68.15% ±0.10
C12	20.27% ±0.10	38.26% ±0.13	53.25% ±0.15	64.58% ±0.14

Data are expressed as mean ±SD (n=3)

T a b l e S5

Factors and responses selected for design of experiments of DL-UMB-CDLP formulation batches

Formulation code	PL:90H Chol ratio	Lipid Drug ratio	Stirring speed	EE (%)	PS (nm)	PDI	ZP (mV)
DL-UMB-CDLP 1	5:5	1:1	800	97.86 ±0.45	243 ±0.07	0.52 ±0.03	-11.79 ±0.83
DL-UMB-CDLP 2	9:1	1:1	800	98.73 ±0.41	142.1 ±0.13	0.50 ±0.01	-8.97 ±1.69
DL-UMB-CDLP 3	5:5	3:1	800	97.61 ±0.39	416.1 ±0.11	0.63 ±0.03	-12.06 ±0.56
DL-UMB-CDLP 4	9:1	3:1	800	96.33 ±0.66	200.4 ±0.15	0.39 ±0.02	-13.01 ±1.47
DL-UMB-CDLP 5	5:5	2:1	600	93.59 ±0.59	151.3 ±0.12	0.35 ±0.04	-9.56 ±0.70
DL-UMB-CDLP 6	9:1	2:1	600	94.82 ±0.54	209 ±0.17	0.58 ±0.04	-8.83 ±0.80
DL-UMB-CDLP 7	5:5	2:1	1000	97.63 ±0.42	210.8 ±0.14	0.53 ±0.10	-21.96 ±0.27
DL-UMB-CDLP 8	9:1	2:1	1000	90.89 ±0.23	180.7 ±0.08	0.62 ±0.07	-9.05 ±1.21
DL-UMB-CDLP 9	7:3	1:1	600	97.06 ±0.72	289.8 ±0.10	0.20 ±0.04	-14.93 ±0.86
DL-UMB-CDLP 10	7:3	3:1	600	97.4 ±0.78	212.9 ±0.80	0.43 ±0.06	-10.26 ±0.89
DL-UMB-CDLP 11	7:3	1:1	1000	96.8 ±0.34	210.8 ±0.28	0.17 ±0.03	-17.43 ±0.64
DL-UMB-CDLP 12	7:3	3:1	1000	95.93 ±0.28	183 ±0.213	0.55 ±0.04	-20.88 ±0.11
DL-UMB-CDLP 13	7:3	2:1	800	99.166 ±0.25	166.3 ±0.40	0.35 ±0.02	-23.6 ±0.49
DL-UMB-CDLP 14	7:3	2:1	800	98.87 ±0.47	169.1 ±0.70	0.51 ±0.02	-8.44 ±0.96
DL-UMB-CDLP 15	7:3	2:1	800	98.65 ±0.64	168 ±0.37	0.33 ±0.05	-22.19 ±0.51

Data are expressed as mean ±SD (n=3)

T a b l e S 6

pH of double-loaded UMB liposomal gel formulations*

	pH of UMB gel
B1	6.73± 0.12
B2	6.92 ± 0.16
B3	6.84 ± 0.2
B4	6.57 ± 0.14

Data are expressed as mean ±SD (n=3)

Table S7

Stability study data of optimised DL-UMB-CDLP

Evaluation Parameters	Optimised Double loaded UMB liposomal formulation						
	Initial (Zero days)	Refrigerator Temperature (4°C ±1°C/ 45%RH)		Room Temperature (25°C ±1°C/ 65%RH)		Accelerated Temp (40°C ±1°C/ 75%RH)	
		30 days	90 days	30 days	90 days	30 days	90 days
EE	99.17% ±0.34	98.87% ±0.03	98.65% ±0.09	98.73% ±0.36	97.86% ±0.54	97.61% ±0.16	96.64% ±0.08
Drug release	81.64% ±1.39	80.83% ±0.89	79.48% ±1.76	78.72% ±0.94	78.33% ±1.25	80.92% ±0.58	77.66% ±1.59

Data are expressed as mean ±SD (n=3)

Table S8

Stability study data of optimised DL-UMB-CDLP loaded gel formulation

Evaluation Parameters	Optimised liposomal UMB GEL formulation						
	Initial (Zero days)	Refrigerator Temperature (4°C ±1°C/ 45%RH)		Room Temperature (25°C ±1°C/ 65%RH)		Accelerated Temp (40°C ±1°C/ 75%RH)	
		30 days	90 days	30 days	90 days	30 days	90 days
pH	6.82 ±0.12	6.76 ±0.14	6.71 ±0.15	6.70 ±0.17	6.68 ±0.19	6.61 ±0.23	6.63 ±0.21
Drug content	98.66% ±0.52	98.51% ±0.45	98.75% ±0.18	97.61% ±0.42	97.53% ±0.10	96.41% ±0.37	95.68% ±0.39
Drug release	93.02% ±0.96	92.71% ±0.56	92.65% ±1.03	92.35% ±0.96	92.08% ±1.65	91.63% ±0.71	90.74% ±0.28
Viscosity	3550 cps ±0.61	3545cps ±0.60	3456cps ±0.46	3545cps ±0.35	3540cps ±0.48	3519cps ±0.64	3267cps ±0.58

Data are expressed as mean ±SD (n=3)

Figure S1. Rat skin images at the end of 7 days of the skin irritancy study where (A)- G1-Gel base, (B)- G2- Placebo, (C)- G3- optimised DL-UMB-CDLP loaded gel

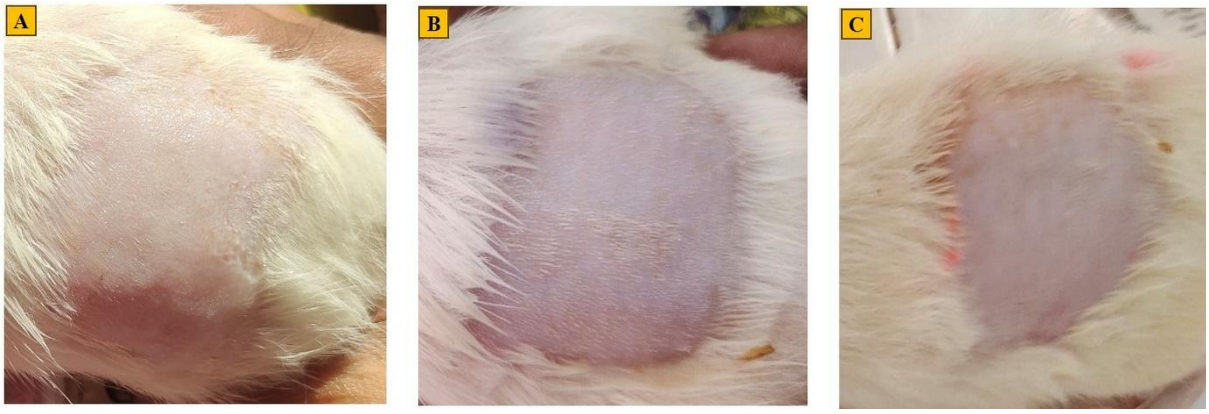


Figure S2. Images of the photoprotective activity where (A)-Gel application on the shaved rat skin, (B, C)-UV light exposure to the rats, (D, E)- Applied gel covered for 4h

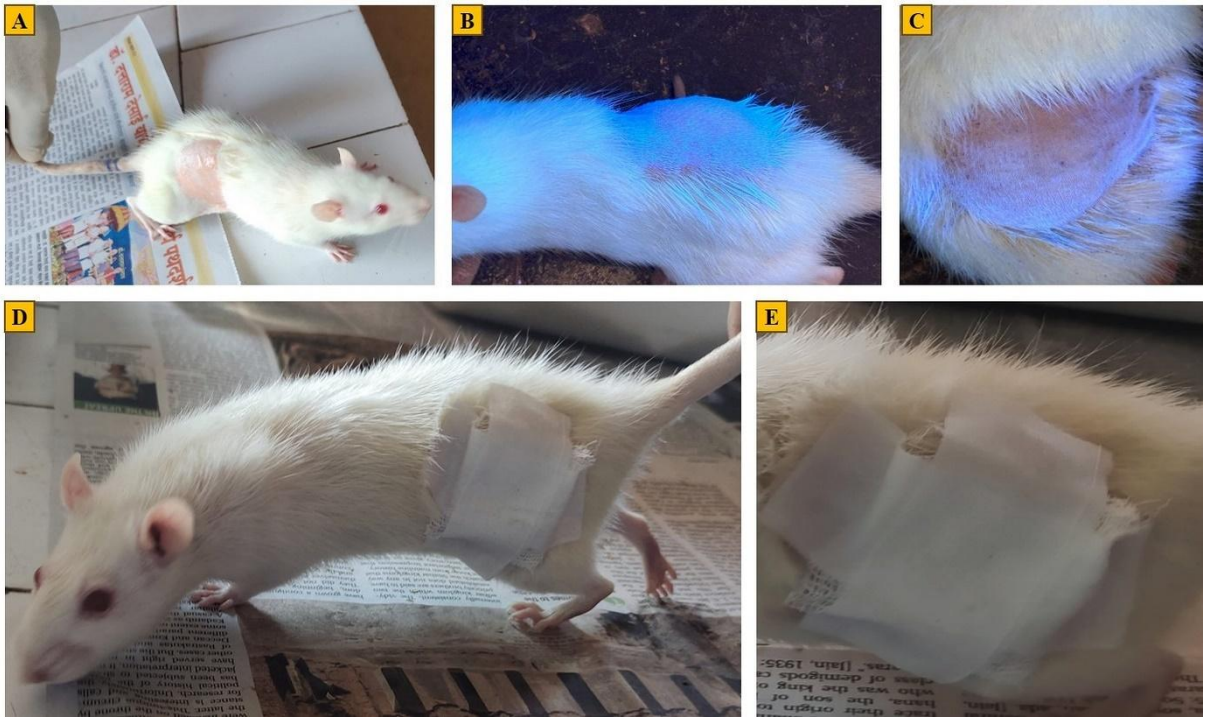
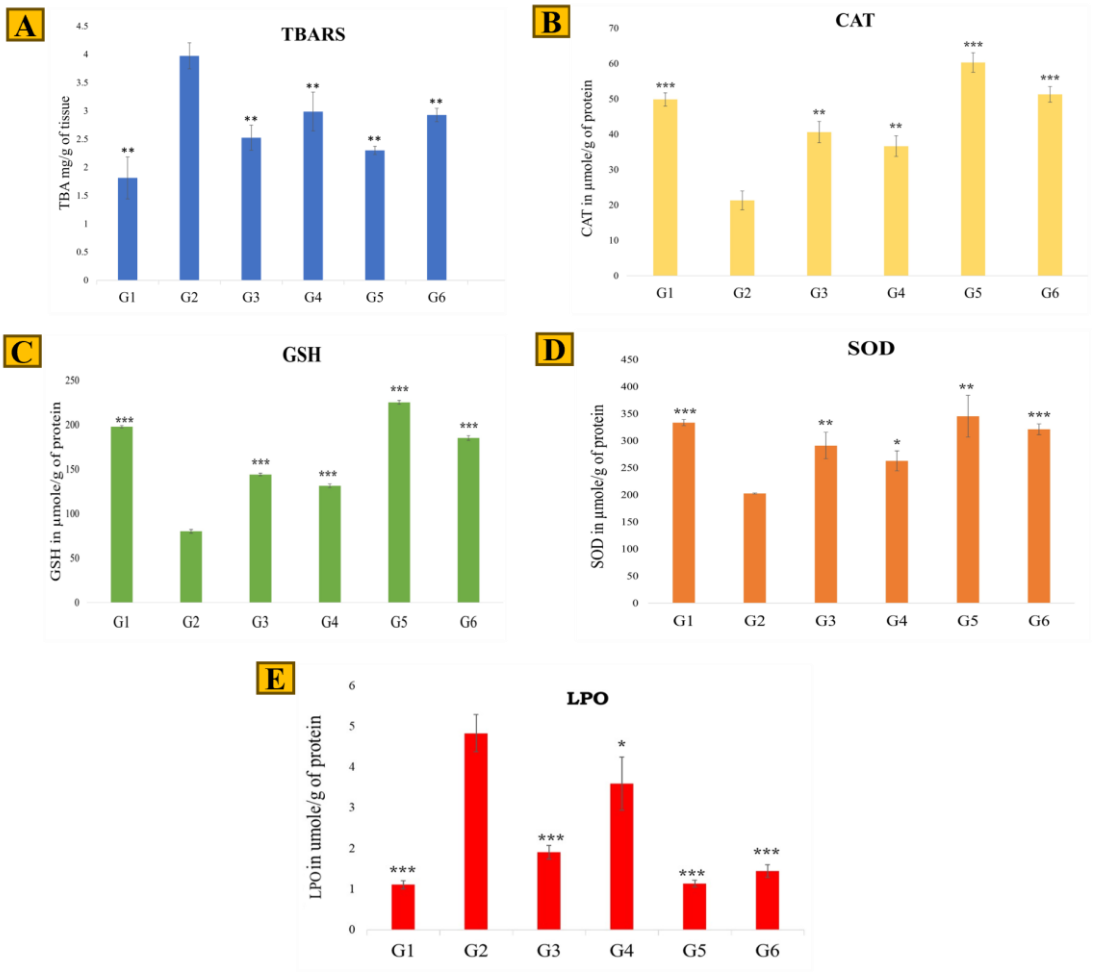


Figure S3. Effect of UV exposure on the antioxidant enzymes: (A) TBARS, (B) Catalase, (C) GSH, (D) SOD, (E) LPO



One-way ANOVA followed by Dunnett's post hoc test. Data represents mean±SD. (n=6), *p<0.01, **p<0.001, and.***p<0.0001 vs G2.