

The Application of a Novel Wrapping Technique for Oleuropein to Improve its Stability and Bioavailability

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1. Introduction

Olive (*Olea europaea* L., Oleaceae), a key Mediterranean crop, is valued for its nutrient-rich products, particularly olive oil. Olive leaves, a major by-product^[1], contain bioactive compounds such as oleuropein (OLE), exhibits antibacterial, anti-inflammatory^[2] and antioxidant properties^{[3][4]}.

However, the application of OLE is constrained. Environmental exposure degrades OLE into unstable hydroxytyrosol (HTY), leading to further degradation and inactivation^[5], resulting in poor stability and color deepening in aqueous OLE solutions. Additionally, OLE's hydrophilicity limits transdermal absorption due to the stratum corneum's lipophilic nature, resulting in low bioavailability^[6].

To overcome the OLE's stability and delivery limitations, researchers have explored various encapsulation and delivery systems. Among these, nanoliposomes have emerged as a promising solution owing to their unique structural characteristics^[7]. Their phospholipid-based structure enables encapsulation of both hydrophilic and hydrophobic compounds, while their biocompatibility, low toxicity, and modifiable surface properties enhance cellular uptake and targeted delivery^[8]. These advantages make liposomes widely used for cosmetic actives^{[9][10]}.

Deep eutectic solvents (DES) have gained recognition as a novel class of functional liquids with broad potential applications. At the molecular level, DES are supramolecular complexes formed through hydrogen bonding, existing as liquids without requiring additional solvents^[11]. A subset of these, natural deep eutectic solvents (NADES) such as betaine (hydrogen bond acceptor) and xylitol (hydrogen bond donor), are commonly found in food products. DES exhibit several advantageous properties, including low flammability, minimal volatility, excellent thermal and chemical stability, wide polarity range, simple synthesis, low vapor pressure, and high solvation capacity^[12].

Here, we developed an innovative delivery system comprising a ternary supramolecular complex (OTS) of OLE, betaine, and xylitol, which was subsequently encapsulated into liposomes (OTSL). This integrated approach was designed to comprehensively address OLE's stability and delivery challenges. The performance of OTSL was evaluated through multiple parameters: skin retention was assessed using Franz diffusion cells; physicochemical properties were characterized by FTIR, MS, and XRD; and a novel LC-MS/MS method was developed for simultaneous quantification of HTY and OLE in olive leaf extracts and cosmetic formulations. Furthermore, the clinical efficacy was validated through a 28-day study involving 35 Chinese subjects with sensitive skin, who used an OTSL-containing mask to evaluate its soothing and repairing effects.

2. Materials and methods

2.1. Materials

Olea europaea (olive) leaf extract (oleuropein≥90%) was obtained from Hunan Aijia Biotechnology Co., Ltd. Phosphatidylcholine was obtained from Phospholipid GmbH (Köln, Germany). Betaine and xylitol were obtained from International Flavors & Fragrances Inc.

The supplier of glycerin was P&G (Ohio, USA). 1,3-propanediol was purchased from DuPont Company (Wilmington, USA). All solvents were of analytical grade and ultrapure water was used to prepare all aqueous solutions. Acetonitrile (LC-MS grade), Methanol (LC-MS grade), Formic acid (LC-MS grade) were purchased from thermo fisher (MA, USA). Ultra-pure water was obtained from a Milli-Q Plus purification system (Millipore, Amsterdam, the Netherlands) was utilized throughout the experiments. SIMULGEL™ FL was obtained from SEPPIC (Paris, France).

2.2. Preparation of OTSL

Betaine and xylitol (1:1 molar ratio) were mixed with a small amount of ultrapure water and heated at 130°C under vacuum (0.06 MPa) for 6 h with stirring. After water evaporation and cooling, a viscous transparent liquid was obtained as a binary supramolecular deep eutectic solvent (DES). *Olea europaea* (olive) leaf extract was dissolved in DES at 45°C to form a homogeneous solution. After cooling to room temperature, a pale yellow, transparent viscous liquid was obtained as the oleuropein-betaine-xylitol ternary supramolecular solution (OTS), which served as the aqueous core phase.

Phospholipids and 1,3-propanediol were completely dissolved at 60°C to obtain a transparent solution. The OTS was then added under continuous stirring at 50°C. This organic phase was injected into an aqueous phase composed of glycerin and purified water (pre-equilibrated at 50°C with stirring). The mixture was homogenized at 15,000 psi for 4 cycles with temperature maintained at 15°C during discharge, yielding yellow-transparent ternary supramolecular olive leaf extract liposomes (OTSL).

3. Characterization

3.1. Particle size and polydispersity index analysis of OTSL

The size (mean hydrodynamic diameter, Dh) and size distribution (polydispersity index, PDI) of liposomes were measured by dynamic light scattering (DLS) techniques, by using instrument of BeNano 180 zeta Pro. The Dh and PDI of the liposomes were measured 24h after sample preparation, as well as for the monitoring of their physical stability over 90 days. DLS measurement was performed at a fixed angle of 173° at 25 °C. The measured data on Dh and PDI were evaluated based on the intensities of the particles, and were assessed in triplicate.

3.2. The concentration of OLE in OTSL analysis

The concentration of OLE in OTSL was analyzed by high-performance liquid chromatography. Oleuropein standard solutions was prepared in methyl: water (with 0.1% phosphoric acid) =50:50 solvent to the concentration of 250.0µg/mL. The OTSL solutions were also diluted to the similar concentration by using same solvent. Then vortex mixed and filtered with 0.45µm filters before injection. The HPLC column (C18

column) temperature was set to 35 °C and the injection volume was 5µL. Gradient elution was performed at a flow rate of 1.0 mL/min. The detailed elution program is shown in Table 1. The detection wavelength is 232 nm. Precision and reproducibility were evaluated by six replicated analyses, and the RSD values were less than 0.2%.

Table 1. The detailed elution program of OLE.

time/min	mobile phase A %	mobile phase B%
	(acetonitrile)	0.1%phosphoric acid aqueous solution
0	15	85
4	25	75
10	30	70
15	30	70

Researches has shown that the stability of oleuropein (OLE) can be affected by various external factors, leading to degrade into unstable hydroxytyrosol (HTY). To determine the content of OLE and HTY in olive leaf extract or cosmetics simultaneously, an LC-MS/MS method was developed. The 78% methanol aqueous solution was used as the solvent. The HPLC column was Poroshell 120 SB - AQ, 4.6mm x 150mm, 2.7µm, with a column temperature of 30°C. Mobile phase A was 0.1% formic acid in water, and mobile phase B was methanol. An isocratic elution mode was adopted, and the flow rate was 0.3 mL/min. The mass spectrometry was run in ESI-negative mode. The data was obtained in multiple reaction monitoring (MRM) mode, and the MRM parameters are shown in Table 2.

Table 2. MRM parameters of OLE and HTY

Component	Precursor ion (m/z)	Product ion (m/z)	Fragmenter(V)	CE (V)
OLE	539.2	307	40	5
		275.1	40	5
HTY	153.1	123.0	110	12
		93.1	110	40

3.3. The skin retention analysis of OTSL

OTSL and Olea europaea (olive) leaf extract water solution (OLES)were separately incorporated into an essence base matrix (composed of 3% 1,3-propanediol, 3% glycerin, 0.8% SIMULGEL FL, with water as balance) and homogenized to prepare test samples at 0.1%, 0.2%, and 0.5% olive leaf extract content. Using Bama piglet skin mounted in Franz cells, the permeation study was conducted at 37°C with 600 rpm stirring. After temperature equilibration, 100 mg samples were applied uniformly, and following 12-hour permeation, oleuropein extraction was centrifuged at 12000 r/min for 10 min. The supernatant obtained was analyzed by HPLC to calculate the OLE retention per unit area of skin.

3.4. Physicochemical properties of OTS analysis

FTIR spectra (ATR) was conducted on InspiritFT-IR (Shimadzu) spectrometer in range of 400–4000 cm⁻¹ wavelengths.

X-ray diffraction (XRD) was used to measure the crystalline structure with an X-ray diffraction equipped with Cu K α radiation source and filtered radiation (wavelength $\lambda = 1.54 \text{ \AA}$) in the scanning range of $2\theta = 5\text{--}80^\circ$ and $10^\circ \text{ min}^{-1}$ scan rate.

Exion AD UPLC and X500R Q-TOF mass spectrometer (Sciex, USA) were used to characterize the Betaine, xylitol, olive leaf extract, and the formulated DES/products. Owing to its high resolution and mass accuracy (<3 ppm), samples were dissolved with purified water and injected into LC-HRMS system for obtain information such as retention time or ions accurate m/z value.

3.5. Human safety and efficacy evaluation of OTSL analysis

A total of 35 participants were recruited for this study. All participants provided informed consent and conducted in accordance with the Declaration of Helsinki in 2024. Exact inclusion and exclusion requirements were established for all volunteers. The eligibility of each participant was evaluated by a dermatologist.

The participants used topical Proya® Double Effect Whitening Brightening Mask (Proya Cosmetics Co., Ltd, China) containing 0.12% OTSL for 28 days. The treatment effectiveness and safety were assessed using objective and subjective methods at baseline, 14 and 28 days. Images were captured using the consistent and standard posture of a VISIA® system (Canfield Scientific, USA) and analyzed skin red area using IPP®. The skin hydration (CORN), trans-epidermal water loss (TEWL), skin tone (a^*), erythema index (EI) were measured by a skin tester (MPA9, Courage+Khazaka electronic GmbH, German). A skilled and experienced dermatologists evaluated the safety and efficacy at each follow-up. All measurements were carried out in the room at temperature 20–22°C, humidity 40–60%. Before each return visit test, subjects were assessed after sitting in the room for 30 minutes.

Statistical analyses were performed by using SPSS version 28.0 software; $p < 0.05$ was considered statistically significant. For the quantitative data, paired Student's t-test and Wilcoxon rank sum test were performed to analyze the differences between the groups; data were shown as means \pm standard deviation. Ranked data were analyzed by using the Wilcoxon rank sum test.

4. Results and discussion

4.1. Particle Size and OLE Content Stability in OTSL

OTSL was prepared by integrating supramolecular DES technology with liposomal encapsulation. DLS measurements showed a mean particle size of 56.51 nm with a narrow PDI of 0.147, confirming monodisperse distribution. The sub-100 nm particle size facilitates effective skin penetration^[14], while the low PDI (<0.3) indicates uniform size, which is essential for maintaining liposomes particle size stability^[13]. The OTSL appeared as a yellow transparent solution that maintained physical stability under different storage conditions for 90 days. The particle size maintained stability with <5 nm size increase. Although a gradual particle size increase was observed at 45°C, due to thermally-induced particle aggregation^[15], the final diameter remained below 100 nm after 90 days.

OTSL, formulated with high-OLE Olea europaea leaf extract, exhibited OLE degradation rates of <3% (room temperature) and <10% (45°C) over 90 days, with no visible changes or detectable odor variation. This represents an improvement

compared to conventional OLE solutions, which typically exhibit over 50% degradation within just 15 days under regular storage conditions (7°C, RT, and 40°C)^[16], confirming that the OTSL preparation process enhanced OLE stability.

4.2. Accumulation of OLE in the skin layers

The skin permeation and retention profile of OLE-containing formulations (OLES and OTSL at 0.1%, 0.2%, and 0.5% OLE) was evaluated using a Franz diffusion cell system with ex vivo Bama piglet skin. For all formulations tested, the amount of OLE recovered in the receptor compartment was under the lower limit of quantification (1 µg/mL), suggesting that the potential systemic absorption was avoided. Quantitative analysis of cutaneous OLE retention demonstrated that the supramolecular encapsulation in OTSL significantly enhanced skin retention capacity ($p < 0.05$), exhibiting a concentration-dependent effect (Figure 1). Notably, at the 0.1% concentration, OTSL showed a 387.68% increase in cutaneous OLE retention compared to conventional OLE formulation.

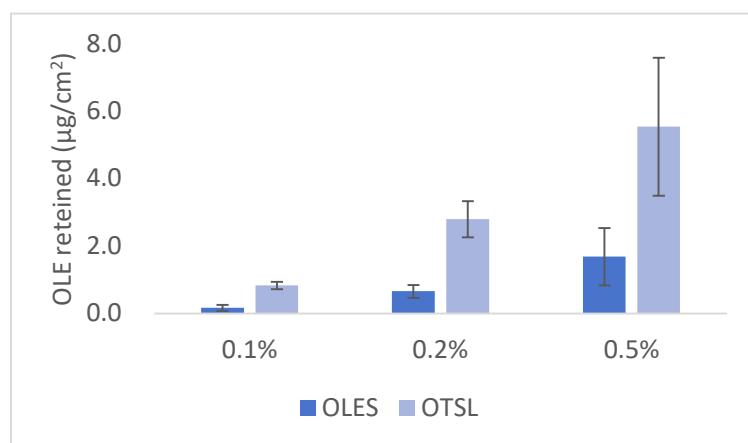


Figure 1. The skin retention of OLE ($\mu\text{g}/\text{cm}^2$) in porcine skin layers after 12 h in-vitro permeation experiment from 0.1%, 0.2%, 0.5% w/w solution of *Olea europaea* (olive) leaf extract (mean values \pm sd).

4.3. Human safety and efficacy evaluation of OTSL analysis

Sensitive skin is often accompanied by different skin irritation symptoms, easy to appear burning, tingling, itching, tightness, with or without erythema and scales^[23]. No subject reported intolerance or allergy to the product during follow-up. Compared to baseline, use of the product for 14 days relieved tingling, tightness, pruritus, burning sensation, and dryness of sensitive skin. After 28 days, the tingling and burning sensation disappeared in all subjects. The detailed elution program is shown in Table 3.

Skin red color response can be used as an indicator of skin sensitivity^[24]. The degree of pre- and post-treatment erythema or redness was measured to analyze soothing and repairing effects of the product containing OTSL by image analysis and 2 different probes (Colorimeter CL400 and Mexameter MX18). Compared to baseline, the red area, a^* and EI significantly decreased after 14 days of product treatment ($P < 0.01$, respectively, Table 4). TEWL and hydration level are usually involved in determining and measuring barrier function^[25]. At the 14th day and 28th day visits, the hydration levels were significantly increased compared to baseline ($p <$

0.001, respectively, Table 3). In contrast to hydration levels, TEWL levels significantly decreased from baseline on days 14 and 28 ($p < 0.001$, respectively, Table 3).

Clinical trial results showed that product containing 0.12% OTSL have a soothing and repairing effect on people with compromised skin barriers.

Table 3 Dermatologist's subjective safety assessment score

Subjective assessment	Baseline (Incidence)	D14 (Incidence)	D28 (Incidence)
Tingling sensation	0.06±0.24 (6%)	0.03±0.17 (3%)	0.00±0.00 (0%)
Tightness sensation	0.57±0.50 (57%)	0.29±0.46 (29%)	0.11±0.32 (11%)
Pruritus sensation	0.20±0.41 (20%)	0.06±0.24 (6%)	0.03±0.17 (3%)
Burning sensation	0.06±0.24 (6%)	0.03±0.17 (3%)	0.00±0.00 (0%)
Drying/desquamation	0.60±0.50 (60%)	0.23±0.43 (23%)	0.17±0.38 (17%)
Erythema	0.00±0.00 (0%)	0.00±0.00 (0%)	0.00±0.00 (0%)
Edema	0.00±0.00 (0%)	0.00±0.00 (0%)	0.00±0.00 (0%)

Scoring criteria: 0 to 3 score, 0 score as "asymptomatic", 1 score as "mild", 2 score as "moderate", 3 score as "obvious". data were shown as means ± standard deviation.

Table 4 The objective evaluation on efficacy

Skin physiological parameters		Baseline	D14	D28
Skin redness	skin red area	39.11%±10.37%	28.83%±9.37%***	26.67%±10.44%***
	a*	11.44±1.47	11.13±1.58**	11.05±1.59***
	EI	288.61±57.18	280.47±57.09***	266.76±57.01***
Skin barrier	CORN	36.37±8.84	46.50±7.77***	55.92±6.64***
	TEWL	17.28±1.45	16.28±1.46***	14.83±1.57***

$p < 0.05$ means significant difference from the baseline, “**” means $p < 0.05$, “***” means $p < 0.01$, “****” means $p < 0.001$.

4.4. OTSL PhysicoChemical Characterization

4.4.1. FTIR spectroscopy

FTIR analysis revealed distinct molecular interactions in the betaine-xylitol DES and OTS systems. For betaine, characteristic vibrations were observed at: 3360.1/3285.4 cm^{-1} (N-C stretching in quaternary ammonium), 1621/1694 cm^{-1} (monomeric/dimeric COOH), and 1614/1487 cm^{-1} (COO- stretching) [20][21]. Xylitol showed typical O-H (3154.7-3423.2 cm^{-1}) and C-OH (1005.1-1124.3 cm^{-1}) vibrations [22]. DES formation was confirmed by: (1) C-OH broadening (1000-1100 cm^{-1}) and new 3000-3600 cm^{-1} hydrogen-bonding network, (2) 10-15 cm^{-1} red-shifted aliphatic C-H vibrations (2882-2932 cm^{-1}), and (3) disappearance of betaine's dimer peak (1694 cm^{-1}) (Figure 2.A).

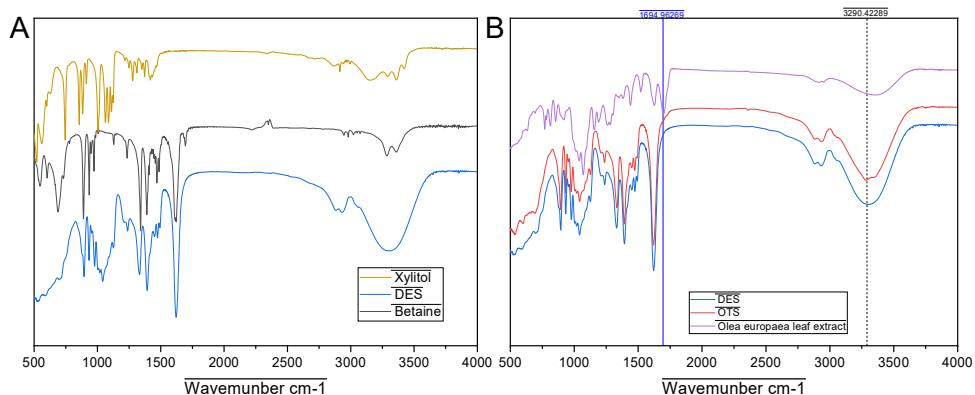


Figure 2. (A) FTIR spectra for Xylitol (Yellow line), Betaine (black line), and Betaine-Xylitol DES (blue line); (B) FTIR spectra for DES (blue line), The Olea europaea leaf extract (purple line), and OTS (red line).

The Olea europaea leaf extract primarily contains iridoid bitter compounds, with oleuropein (OLE) being the most bioactive constituent. Spectroscopic analysis of the OTS revealed intermolecular interactions: the disappearance of the 1695 cm⁻¹ dimer peak confirmed hydrogen bonding, while the emergence of a new 3290 cm⁻¹ absorption indicated integration of OLE's glycosyl moiety into the xylitol-based hydrogen-bonding network via polyhydroxy structural matching. These findings collectively demonstrate the hierarchical assembly from binary DES to ternary supramolecular complexes (Figure 2.B).

4.4.2. X-ray diffraction

Figure 3 presents the comparative XRD patterns of OTS and its precursors. The sharp diffraction peaks observed for betaine and xylitol confirm their polycrystalline nature, while OLE exhibits characteristic semicrystalline features evidenced by broadened peaks and reduced intensity, attributable to the structural flexibility of its glycosylated secoiridoid skeleton and hydrogen bonding. It is clear that the characteristic peaks of betaine and xylitol are disappeared in the XRD pattern of the OTS, confirmed the amorphous nature of OTS, indicating that hydrogen-bonding interactions inhibit the crystallization between betaine and xylitol.

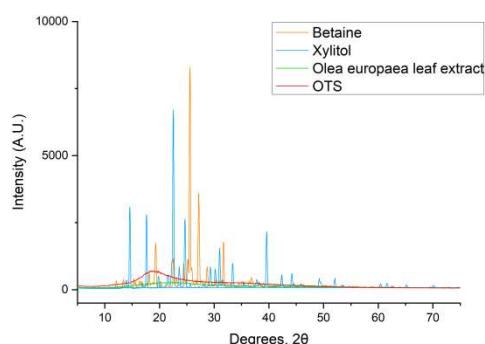


Figure 3. X-ray diffraction pattern of betaine (orange line), Xylitol (blue line), Olea europaea leaf extract (green line) and OTS (red line).

4.4.3. MS Spectrometric Analysis

Mass spectrometric characterization was performed using extracted ion chromatograms (XIC) and high-resolution MS (HRMS) for precise molecular weight and isotopic pattern analysis. Xylitol exhibited excellent mass accuracy with $[M+Na]^+$ at m/z 175.0575 (mass error: 1.1 ppm) (Figure 4.A). For betaine, the observed $[M+H]^+$ ion at m/z 118.0860 showed a minimal mass error of 2.2 ppm compared to theoretical value, confirming its identity (Figure 4.B).

The olive leaf extract, being a complex mixture, was primarily analyzed for oleuropein (OLE) identification. The predominant $[M+H]^+$ adduct at m/z 541.1915 (mass error: 0.1 ppm) confirmed OLE presence (Figure 4.C). In the OTS system, EIC profiles clearly revealed the coexistence of xylitol (RT: 1.227 min), betaine (RT: 1.301 min) and OLE (RT: 4.506 min). HRMS analysis confirmed the hydrogen-bonded supramolecular architecture of OTS through reversible assembly without covalent bond formation (Figure 4.D).

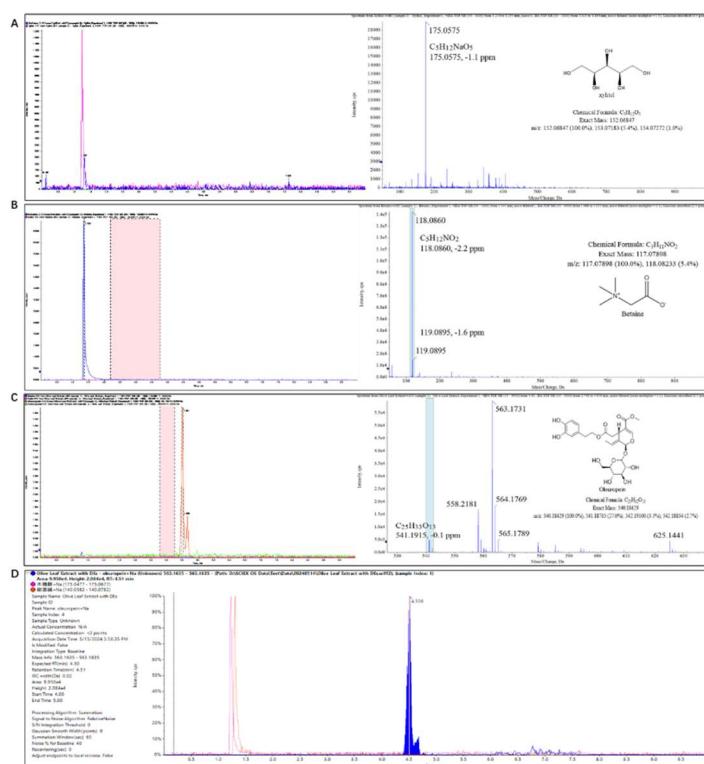


Figure 4. MS Spectra of Xylitol, Betaine, Olea europaea Leaf Extract and OTS:(A-C)
Extracted ion chromatogram (XIC) and high-resolution MS spectrum of xylitol,
betaine and Olea europaea leaf extract; (D) Total ion chromatogram of OTS.

4.5 Simultaneous determination of OLE and HTY by LC-MS/MS

By adjusting test parameters of LC-MS/MS, the method for simultaneous determination of oleuropein and hydroxytyrosol in olive leaf extract or cosmetics was developed. As shown in Figure 5 (TIC chart), the retention time of hydroxytyrosol was 4.306 min, and that of oleuropein was 17.591 min. To ensure the applicability of the method, methodological validation was conducted, including linearity, precision, reproducibility, LOD, LOQ, etc. The experimental results showed that this method has the advantages of high accuracy and wide applicability.

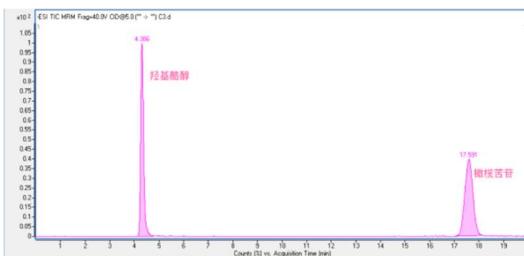


Figure 5. TIC Spectra of OLE and HTY

5. Conclusions

Astrid E. Delorme et al. demonstrated that optimizing the composition, molar ratio, and concentration of natural deep eutectic solvents can significantly enhance the thermal stability of laccase^[12]. Building upon this finding, we developed a ternary supramolecular liposomal system (OTSL) combining DES technology with nanoliposome encapsulation. The observed stability enhancement of oleuropein (OLE) in OTSL formulations confirmed that supramolecular solvent systems can effectively protect bioactive compounds from thermal degradation. The OTSL system offered enhanced stability, improved skin retention, preserved bioactivity, and skin barrier-repairing properties.

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