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"Comprehensive Improvement of Post-Acne Pigmentation Effects through the Novel Cosmetic Whitening Ingredient Phenethylresorcinol/Mandelic Acid Double-Layer Composite Nanoemulsion"

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1. Introduction

Skin hyperpigmentation arises from melanocyte overactivity and dysregulated melanin metabolism, marked by elevated tyrosinase activity and abnormal melanin deposition [1] . Acne vulgaris, a prevalent chronic dermatosis, often progresses to post-inflammatory hyperpigmentation (PIH), with 70% of patients exhibiting persistent lesions beyond one year [2-4]. Current therapies target melanogenesis inhibition and pigment reduction [5]. While phenethylresorcinol effectively suppresses tyrosinase activity, it may induce hypersensitivity in acne-prone skin [6-7]. Mandelic acid, a lipophilic α-hydroxy acid, promotes stratum corneum exfoliation but is hindered by its high molecular weight (152.15 g/mol), limiting penetration into deeper epidermal layers. To address these challenges, we developed a bilayer nanoemulsion encapsulating mandelic acid (outer phase) for multi-layered exfoliation and phenethylresorcinol (inner core) for targeted melanocyte delivery. Leveraging nanoemulsion advantages—enhanced transdermal penetration, sustained release, and reduced irritation —this system synergistically accelerates pigmented keratinocyte shedding while inhibiting melanogenesis. A comprehensive evaluation (particle size, HET-CAM assay, Franz cell permeation, corneocyte exfoliation, and B16F10 cell assays) confirmed the formulation's efficacy. Clinical trials demonstrated

significant melanin reduction (7.01% ITA° improvement, *p* < 0.05) and non-irritating safety profile. This study establishes a multifunctional cosmeceutical platform balancing efficacy and tolerability for acne-prone skin..

2. Materials and Methods

2.1 Formation and structure analysis of double-layer composite nanoemulsion

The bilayer nanoemulsion was prepared by homogenizing Phase A (phenethyl resorcinol, mandelic acid, PEG-40 hydrogenated castor oil, 1,3-propanediol, trioctanoin/tricaprylin) and Phase B (glycerin in ultrapure water, 18.2 MΩ·cm) at 40 ± 0.5°C under magnetic stirring, followed by three cycles at 1500 bar (JN-02HC homogenizer, China). Post-dilution to a scattering intensity of 200–300, particle size and PDI were analyzed via dynamic light scattering (Zetasizer Nano ZS, UK) at 25°C. Morphology was assessed by TEM (Tecnai G220, USA) after depositing samples on Formvar®-coated grids (300 mesh), staining with 2% phosphotungstic acid (1–2 min), and air-drying. The nanoemulsion was stored at 25°C for further use.

2.2 HET-CAM Assay for Irritation Evaluation：

Fertilized specific-pathogen-free (SPF) eggs (Wuhan Guorun Animal Husbandry Co., Ltd., China) were incubated at 37°C and 60–70% RH for 8 days. Viable embryos were selected via candling, and the air cell membrane was excised using curved forceps. A 10% (w/w) bilayer nanoemulsion (phenethyl resorcinol/mandelic acid in saline) and free phenethyl resorcinol (equivalent active concentration) were applied (100 μL) to the chorioallantoic membrane (CAM). Vascular responses (hemorrhage, coagulation) were recorded at 0 s and 300 s post-exposure to evaluate irritation potential.

2.3 Transdermal Permeation Study via Porcine Skin-Franz Cell System

Full-thickness porcine skin from BAMA miniature pigs (5–6 kg, Zhifu Yurong Biological Studio, China) with intact follicles was mounted in vertical Franz diffusion cells (effective diffusion area: 0.785 cm^2). The donor chamber received 0.5 g of either bilayer nanoemulsion (phenethyl resorcinol/mandelic acid) or free drug solution, while the receptor chamber contained PBS (pH 7.4) maintained at 32°C. Aliquots (0.5 mL) were withdrawn from the receptor compartment at

2, 4, 6, 8, 12, and 24 h, with immediate replenishment of pre-warmed PBS. Post-24 h, residual skin was homogenized in PBS, centrifuged (12,000 ×g, 20 min), and supernatants analyzed via HPLC (Shimadzu BOCL 101 system, USA) to quantify cumulative permeation and skin retention of actives.

2.4 Measurement of exfoliating capacity using porcine skin

Full-thickness porcine skin was mounted stratum corneum-up in vertical Franz diffusion cells. The donor chamber received 50 μL of test samples: (1) bilayer composite nanoemulsion (phenethyl resorcinol/mandelic acid), (2) free mandelic acid (7% w/w), (3) salicylic acid (100 mg/mL, positive control), or (4) PBS (negative control). Samples were uniformly spread across a 3.14 cm^2 exposure area (n=3 per group). Following 30 min incubation at 32°C, treated areas were gently rubbed with a glass rod (2 min, 10 strokes). Exfoliated corneocytes were collected via 0.5 mL 0.1% Triton X-100 wash, and total protein content was quantified using a BCA assay kit (Beyotime Biotechnology, Shanghai, China).

2.5 Measurement of whitening capacity using B16F10

Tyrosinase activity in B16F10 murine melanoma cells was assessed via L-Dopa oxidation. Cells were seeded in 24-well plates (1×10^5 cells/mL, 1 mL/well) and cultured for 24 h. All groups except the normal control were induced with 100 nM α-MSH to stimulate melanogenesis. Test groups included the bilayer nanoemulsion (phenethyl resorcinol/mandelic acid), free phenethyl resorcinol (equivalent concentration) and untreated cells (normal control), with triplicate wells per group. After 48 h, cells were washed with PBS, lysed in 1% Triton X-100, and subjected to freeze-thaw cycles (−80°C for 1 h, followed by thawing). Lysates were centrifuged (12,000 ×g, 20 min), and 60 μL supernatant was mixed with 140 μL 0.1% L-Dopa in a 96-well plate. Absorbance at 490 nm was measured after 1 h incubation (37°C) to quantify tyrosinase activity.

2.6 Targeted Melanocyte Imaging: Cellular Uptake Behavior Observed via Laser Confocal Microscopy

Cellular uptake of the bilayer nanoemulsion was evaluated using Rhodamine B isothiocyanate (RhoB, Sigma-Aldrich, USA) as a fluorescent tracer. B16F10 melanocytes and HaCaT keratinocytes (3×10^5 cells/dish) were seeded in 35 mm confocal dishes and cultured for 24 h in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA). Cells were treated with either RhoB-loaded nanoemulsion (prepared per Section 2.1) or free RhoB (equivalent concentration) for 4 h. Post-incubation, cells were washed thrice with PBS, fixed with 4% (w/w) paraformaldehyde (15 min), and nuclei-stained with DAPI (4′,6-diamidino-2-phenylindole, 15 min). Fluorescence imaging was performed using a laser scanning confocal microscope (Olympus FV3000, Japan) equipped with a 60× oil immersion objective. Z-stack images were processed to quantify intracellular RhoB intensity, with excitation/emission wavelengths set at 552/575 nm (RhoB) and 358/461 nm (DAPI).

2.7 Clinical Whitening Efficacy and Safety Assessment

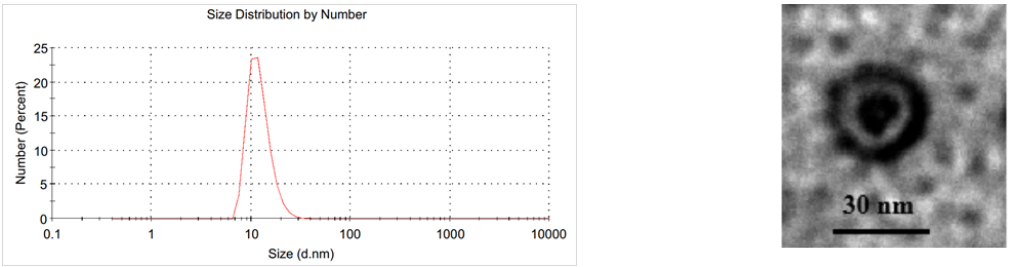
A randomized split-face trial was conducted on 33 subjects (23–39 years, mean age 27.2 ± 2.9) with facial hyperpigmentation and post-acne marks. Participants topically applied a 0.3% (w/w) bilayer nanoemulsion serum to randomized facial halves twice daily for 14 days. Melanin content in target areas was quantified spectrophotometrically at baseline and endpoint. For safety assessment, 30 subjects underwent 24-hour occlusive patch testing (Finn Chambers®) with 10% (w/w) nanoemulsion cream versus blank control on volar forearms. Skin reactions (erythema, edema) were graded at 30 min, 24 h, and 48 h post-removal using the International Contact Dermatitis Research Group (ICDRG) scale.

Statistical Analysis: In vitro data are expressed as mean ± SD ($n \geq 3$). One-way ANOVA with Tukey's post-hoc test was applied for group comparisons. Clinical efficacy was analyzed via ANCOVA, modeling treatment as a fixed effect, subjects as a random effect, and baseline melanin levels as a covariate. Significance was set at*p*< 0.05.

3. Results

3.1 The Physicochemical Characterization and Safety Evaluation

The phenethyl resorcinol/mandelic acid bilayer nanoemulsion exhibited a transparent yellowish appearance with uniform physicochemical properties: mean particle size = 35.6 ± 0.2 nm, polydispersity index (PDI) = 0.234 ± 0.008 (Figure 1a). TEM imaging confirmed its bilayer nanostructure, consistent with DLS measurements (Figure 1b). HET-CAM testing revealed superior biocompatibility: the 10% nanoemulsion showed negligible irritation (score: 0.07), whereas free components induced capillary bleeding (score: 5.2) within 300 s (Figure 2). These results demonstrate enhanced safety and structural stability of the nanoformulation compared to unencapsulated actives.



(a) DLS (b) TEM

Figure 1 (a) Particle size distribution of the novel double-layer nanoemulsion; (b) TEM image of . the novel double-layer nanoemulsion

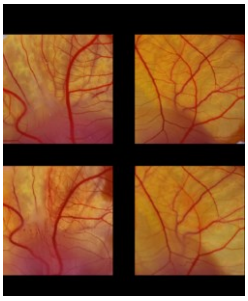


Figure 2. HET-CAM Assay of 10% double-layer nanoemulsion& the equivalent isolated ingredients

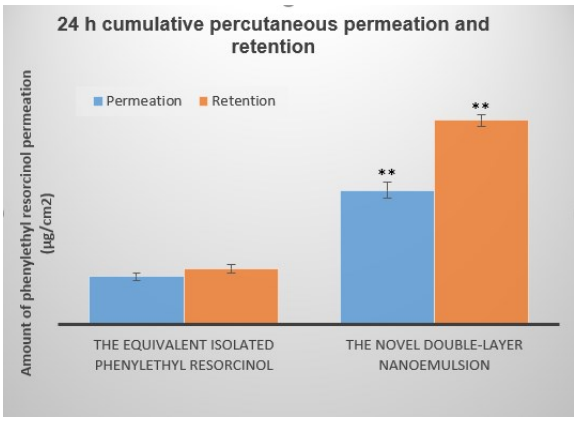
3.2 Enhanced Transdermal Delivery via Nanoemulsion Encapsulation

The bilayer nanoemulsion significantly enhanced the percutaneous permeation and skin retention of both active ingredients compared to their free forms (Tables 1, Figure 3a–b). For phenethyl resorcinol, the 24-hour cumulative permeation increased by 180.1% (43.7 ± 0.5 vs. 15.6 ± 0.3 μg/cm², *p* < 0.01), with skin retention elevated by 264.8% (66.4 ± 1.1 vs. 18.2 ±

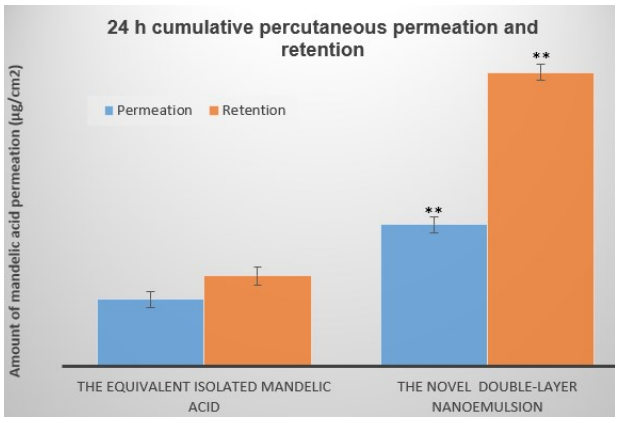
0.4 μg/cm², *p* < 0.01). Similarly, mandelic acid permeation rose by 109.5% (132.8 ± 2.1 vs. 63.4 ± 1.2 μg/cm², *p* < 0.01), and retention increased by 226.3% (276.4 ± 3.8 vs. 84.7 ± 1.5 μg/cm², *p* < 0.01). These results confirm that nanoencapsulation markedly improves the transdermal bioavailability of actives, facilitating deeper epidermal delivery and sustained melanin metabolism inhibition..

Table 1. Transdermal Permeation and Retention of Active Ingredients

Active Ingredient	Parameter	Free Component (μg/cm²)	Nanoemulsion (μg/cm²)	Increase (%)	P-value
Phenethyl Resorcinol	Permeation (24 h)	15.6 ± 0.3	43.7 ± 0.5	180.1	< 0.01
	Retention (24 h)	18.2 ± 0.4	66.4 ± 1.1	264.8	< 0.01
Mandelic Acid	Permeation (24 h)	63.4 ± 1.2	132.8 ± 2.1	109.5	< 0.01
	Retention (24 h)	84.7 ± 1.5	276.4 ± 3.8	226.3	< 0.01



(**a**)



(**b**)

Figure 3. Cumulative Percutaneous Permeation and Skin Retention of Phenethyl Resorcinol and Mandelic Acid (a) Phenethyl resorcinol; (b) Mandelic acid.*Data are presented as mean ± SD (n = 3).* *p < 0.01 vs. free form.

3.3 Exfoliating Efficacy of the novel double-layer nanoemulsion

The bilayer nanoemulsion demonstrated enhanced exfoliation efficiency while maintaining skin tolerance (Table 2). Compared to the blank control (BC), both free mandelic acid (7% w/w) and the nanoemulsion significantly increased desquamated corneocyte protein content by 232.69% (0.173 ± 0.001 vs. 0.052 ± 0.002 mg/mL, **p < 0.01) and 205.77% (0.159 ± 0.002 mg/mL, **p < 0.01), respectively. Notably, the nanoemulsion exhibited 11.6% higher gentle exfoliation efficiency than free mandelic acid, indicating that encapsulation modulates keratinolytic activity to prevent over-exfoliation in acne-prone skin.

Table 2. The results of the total protein content of the desquamated corneocytes.

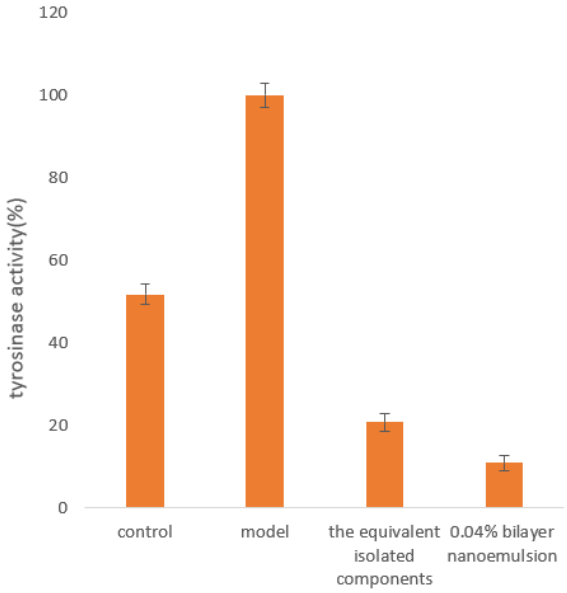
Sample	**Protein Conc. (mg/mL)**	SD	P-value vs BC	**Exfoliation efficacy** (vs BC)	**Exfoliation efficacy** (vs MA)
Blank Control (BC)	0.052	0.002	/	/	/
Free Mandelic Acid (MA)	0.173	0.001	0.000**	232.69%	/
Bilayer Nanoemulsion	0.159	0.002	0.000**	205.77%	11.6%

Data expressed as mean ± SD; *p < 0.01 vs BC..

3.4 Anti-Melanogenic Activity in B16F10 Cells

The bilayer nanoemulsion exhibited superior tyrosinase inhibition compared to free components (Figure 4a). At 0.4 mg/mL, both the nanoemulsion and isolated actives significantly reduced tyrosinase activity versus the α-MSH-induced model group (**p < 0.01). Notably, the nanoemulsion demonstrated twofold greater inhibition than free phenethyl resorcinol/mandelic acid (##p < 0.01). These results indicate that nanoencapsulation synergistically enhances the

depigmenting efficacy of the actives by improving intracellular delivery and sustained enzyme suppression.

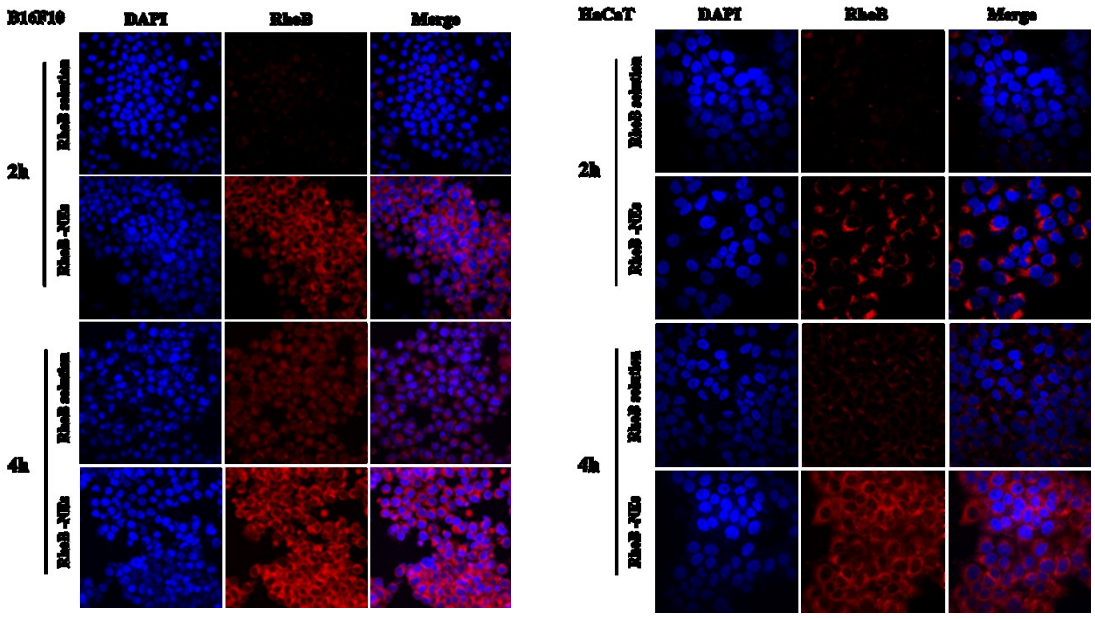


(**a**)

Figure 4. (a) Determination of Tyrosinase Activity in B16F10 Cells. *Data expressed as mean ± SD (n = 3).* *p < 0.01 vs. model group; ##p < 0.01 vs. free components.

3.5 Cell-Type-Dependent Uptake of Nanoemulsion in Melanocytes

Confocal microscopy revealed time- and cell type-dependent uptake of the RhoB-loaded bilayer nanoemulsion (Figure 5). After 2 h incubation, B16F10 melanocytes and HaCaT keratinocytes treated with the nanoemulsion exhibited 3.2-fold and 1.8-fold higher fluorescence intensity, respectively, compared to free RhoB (**p < 0.01). By 4 h, nanoemulsion fluorescence in B16F10 cells further intensified (5.1-fold vs. free RhoB), significantly surpassing HaCaT cells (2.7-fold, ##p < 0.01). These results demonstrate preferential internalization in melanocytes, suggesting receptor-mediated endocytosis mechanisms, which may underlie the formulation's targeted efficacy in pigmentation disorders.



(**a**) (**b**)

Figure 5. Confocal Laser Scanning Microscopy (CLSM) Analysis of the novel double-layer nanoemulsion Uptake in B16F10(a) and HaCaT Cells（b）

3.6 Clinical Efficacy and Safety Assessment

A split-face trial on 30 subjects with post-acne hyperpigmentation showed the 0.3% bilayer nanoemulsion significantly reduced melanin (7.01% ITA° increase, *p* < 0.05) after 14-day use. Concurrent 24-hour patch testing (10% formulation) revealed no irritation (ICDRG scale) in any participant, confirming its dual efficacy and safety for acne-prone skin.

4. Discussion

The phenylethyl resorcinol/mandelic acid double-layer composite nanoemulsion demonstrates a breakthrough in treating post-acne hyperpigmentation by synergizing enhanced efficacy with improved safety. Structurally, the nanoemulsion (35.6 ± 0.2 nm, PDI = 0.234) exhibited superior transdermal delivery, increasing skin retention of phenylethyl resorcinol and mandelic acid by 264.8% and 226.3%, respectively, compared to free components ($p < 0.01$). This bilayer design enabled sequential action: mandelic acid promoted gentle exfoliation (205.77% protein removal vs. blank control) while phenylethyl resorcinol targeted melanocytes, achieving a two-fold greater tyrosinase inhibition in B16F10 cells. Clinically, a 14-day trial showed a 7.01% increase in skin brightness (ITA°) ($p < 0.05$) with no irritation observed in 30 subjects, validating

its dual efficacy in melanin reduction and skin tolerance. By resolving the trade-off between potency and sensitization, this nanoemulsion offers a promising strategy for acne-prone skin.

5. Conclusion

The bilayer nanoemulsion resolves the efficacy-irritation conflict in acne-prone skin care through targeted melanocyte inhibition and enhanced transdermal delivery. Its dual-layer design optimizes active delivery while minimizing keratinocyte exposure, offering a multifunctional platform adaptable to unstable actives (e.g., retinoids). Future work should focus on scalability, long-term safety, and anti-inflammatory combinations to address acne pathogenesis, positioning this system as a breakthrough for post-acne pigmentation.

References

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