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"Investigation on Skin Penetration and Anti-inflammatory Repair of Paeonol/Hydroxy-asparaginamide/Heparin Sodium/ Bisabolol/Glycyrrhizic Acid Nanocarriers"

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1. Introduction

The skin barrier is a vital physiological structure that safeguards against environmental aggressors, pathogens, and transepidermal water loss, while maintaining nutrient homeostasis [1]. Its dysfunction is a hallmark of inflammatory skin disorders such as eczema, psoriasis, and acne, often exacerbating oxidative stress and immune dysregulation. Natural bioactive compounds like paeonol (from Paeonia suffruticosa) have garnered attention for their multi-target anti-inflammatory, antimicrobial, and antioxidant activities, as demonstrated in both in vitro and clinical models [2–3]. Similarly, madecassoside, a triterpenoid from Centella asiatica, accelerates wound healing and mitigates inflammatory markers like IL-6 and TNF-α, making it a cornerstone in dermatological therapies [4]. Heparin sodium, beyond its anticoagulant role, modulates inflammatory pathways by neutralizing chemokines and enhancing tissue repair [5]. Despite their therapeutic potential, these agents face challenges in conventional formulations, including poor skin permeability, instability, and short retention times, which limit their clinical efficacy .Recent advancements in nanotechnology offer transformative solutions. Nanocarriers, such as lipid-based nanoparticles and polymeric micelles, enhance drug solubility, protect labile compounds from degradation, and enable sustained release, thereby optimizing therapeutic outcomes[6-7]. For instance, nanoemulsions co-delivering salicylic acid and antimicrobial

peptides have shown superior skin penetration and prolonged antibacterial activity compared to free formulations[9]. Transdermal co-delivery systems further leverage synergistic interactions between multi-mechanistic compounds—such as combining anti-inflammatory agents with barrier-repair molecules—to address complex dermatological conditions holistically[8]. Studies highlight that nanocarriers encapsulating anti-inflammatory actives significantly reduce pro-inflammatory cytokines (e.g., IL-1α, PGE_2) while enhancing the expression of barrier proteins like filaggrin and claudin-1 in epidermal models. However, integrating plant-derived actives with distinct pharmacological profiles into a unified nanoplatform remains underexplored, particularly for inflammatory and barrier-deficient skin.

This study addresses these gaps by engineering a co-delivery nanocarrier system incorporating paeonol, madecassoside, and heparin sodium. By harnessing nanotechnology, we aim to overcome bioavailability limitations, amplify synergistic anti-inflammatory effects, and prolong cutaneous retention. This approach not only aligns with the demand for advanced, multi-functional cosmeceuticals but also provides a scalable strategy to enhance therapeutic precision in managing inflammatory skin diseases, bridging the gap between natural bioactive efficacy and clinical application.

2. Materials and Methods

2.1 Material and Instruments

The following materials and instruments were utilized: paeonol (Shaanxi Huike Plant Development Co., Ltd.), madecassoside (Seppic, France), heparin sodium (Hebei Changshan Pharmaceutical Co., Ltd.), pentanediol (B&B, Korea), hexanediol (Inolex, USA), trioctanoin/decanoin glycerides (Croda, UK), polyglyceryl-4 oleate (Nikkol Chemicals, Japan), DMEM high-glucose medium, fetal bovine serum (FBS), penicillin-streptomycin (Gibco, USA), ELISA kits for filaggrin (FLG), aquaporin-3 (AQP3), claudin-1, interleukin-1α (IL-1α), IL-6, and prostaglandin E2 (PGE_2) (Jiangsu Meimian Industrial Co., Ltd.), rhodamine B, and fluorescein isothiocyanate. Instruments included a high-speed shear machine (IKA, Germany), Zetasizer/Nano-ZS90 nanoparticle analyzer (Malvern, UK), BOCL 101 HPLC system (Shimadzu, Japan), TPY-

2 transdermal diffusion apparatus (Shanghai Huanghai Pharmaceutical Instrument Co., Ltd.), multimode plate reader (PerkinElmer, USA), laminar flow hood (Suzhou Antai Group), BB150 CO_2 incubator (Thermo Fisher Scientific, USA), and FV3000 confocal laser scanning microscope (Olympus, Japan).

2.2 Preparation and characterization of Nanocarriersnts

Paeonol, trioctanoin/decanoin glycerides, and polyglyceryl-4 oleate (Phase A) were dissolved at 50°C, while pentanediol and hexanediol (Phase B), along with madecassoside, heparin sodium, and ultrapure water (Phase C), were separately heated to 50°C. The three phases were combined, homogenized by stirring for 30 min, and processed using a high-speed shear machine to form nanocarriers. The particle size, polydispersity index (PDI), and zeta potential of the nanocarriers were analyzed after dilution with ultrapure water using a Zetasizer/Nano-ZS90 at 25°C, with particle size measurements performed at a 90° scattering angle.

2.3 Skin Permeation and Retention：

Excised porcine skin was mounted in Franz diffusion cells. Free active compounds or nanocarrier formulations (0.5 g) were applied to the donor chamber. The receptor medium (5% Tween 80, 25% propylene glycol, and 70% saline) was maintained at 37°C. Samples (0.5 mL) were collected at 2, 4, 6, 8, 10, 12, and 24 h, with fresh medium replenished. After 24 h, skin was homogenized, centrifuged, and analyzed by HPLC to quantify paeonol permeation and retention.

2.4 Confocal Microscopy for Skin Penetration

Rhodamine B (RhoB)-loaded nanocarriers or free RhoB (0.5 g) were applied to porcine skin in Franz cells. After 2 and 4 h, skin sections were rinsed, frozen, sliced, and imaged using a confocal microscope.

2.5 Skin Barrier Repair Marker Analysis

HaCaT cells (1×10^5 cells/mL) were cultured in 24-well plates for 24 h. Free madecassoside or nanocarriers were added. After 24 h, supernatants were assayed for FLG, AQP3, and claudin-1 via ELISA.

2.6 Anti-inflammatory Activity

RAW264.7 cells (2×10^5 cells/mL) were cultured and stimulated with LPS (1 μg/mL). Free madecassoside or nanocarriers were added. IL-1α, IL-6, and PGE_2 levels in supernatants were measured after 24 h.

2.7 Statistical Analysis

Data were expressed as mean ± SD and analyzed using SPSS 20.0. One-way ANOVA with post-hoc tests assessed significance ($p < 0.05$).

3. Results

3.1 The Physicochemical Characterization

The nanocarriers exhibited a light-yellow transparent appearance, with drug loading capacities of 1% for paeonol, 2% for madecassoside, and 0.1% for heparin sodium. Characterization using a nanoparticle size analyzer revealed a hydrodynamic diameter of 35.8 ± 0.7 nm, a polydispersity index (PDI) of 0.151 ± 0.002, and a zeta potential of 28.98 ± 0.20 mV, indicating a monodisperse and stable colloidal system.

3.2 Skin Permeability

As shown in Figure 1, the cumulative skin permeation of free paeonol and paeonol-loaded nanocarriers (at equivalent doses) over 24 h was 22.2 ± 1.5 μg/cm² and 49.1 ± 2.1 μg/cm², respectively, while their skin retention values were 7.1 ± 0.6 μg/cm² and 13.8 ± 0.9 μg/cm². Compared to free paeonol, the nanocarrier formulation enhanced cumulative skin permeation by 116.3% and skin retention by 124.5%, demonstrating significantly improved transdermal delivery and sustained retention of the active compound.

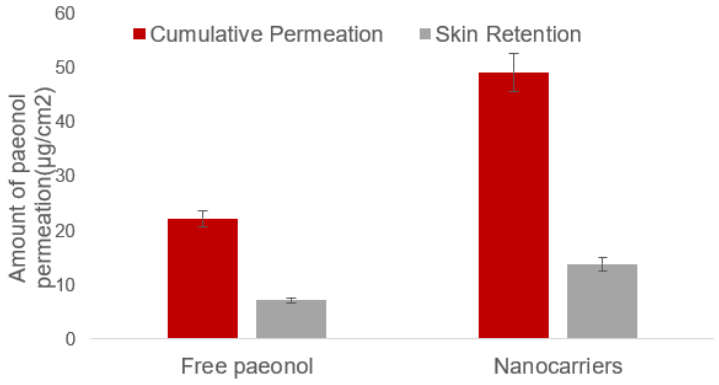


Figure 1. Comparative skin permeation and retention profiles of free paeonol versus nanocarrier-encapsulated paeonol. *p < 0.01 vs. free paeonol.

3.3 Confocal Microscopy for Skin Penetration

The skin penetration behaviors of free Rhodamine B (RhB) and RhB-loaded nanocarriers were analyzed via confocal laser scanning microscopy (Figure 2). As depicted in Figure 2, free RhoB predominantly localized within the stratum corneum at 2 h, demonstrating minimal penetration through the epidermal barrier. In contrast, RhoB co-delivery nanocarriers exhibited robust trans-barrier permeation by 2 h, with fluorescence intensity markedly increasing in the deeper dermal layers by 4 h. These findings confirm that nanocarrier encapsulation significantly enhances the delivery efficiency of active compounds to deeper skin tissues.

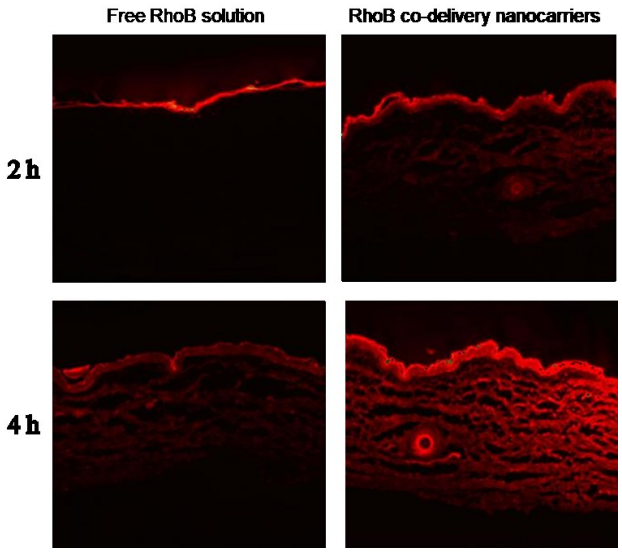
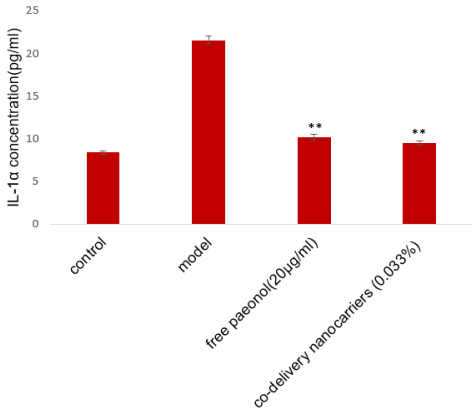


Figure 2. Confocal microscopy analysis of cutaneous permeation dynamics.

3.4 Anti-Inflammatory

Lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages exhibited significantly elevated secretion of inflammatory mediators, including interleukin-1α (IL-1α), IL-6, and prostaglandin E2 (PGE_2). As shown in Figure 3, both free active compounds and nanocarrier formulations markedly suppressed LPS-induced cytokine release. Compared to the LPS-treated model group, nanocarriers reduced IL-1α, IL-6, and PGE_2 levels by 48.2% ($p < 0.001$), 52.7% ($p < 0.01$), and 41.9% ($p < 0.05$), respectively. Notably, at equivalent doses, nanocarriers demonstrated superior anti-inflammatory efficacy over free compounds, achieving additional reductions of 22.3% ($p < 0.05$) for IL-1α, 18.6% ($p < 0.05$) for IL-6, and 15.8% ($p < 0.01$) for PGE_2 (Figure 3a–c). These data validate the enhanced anti-inflammatory activity of nanocarrier-encapsulated therapeutic



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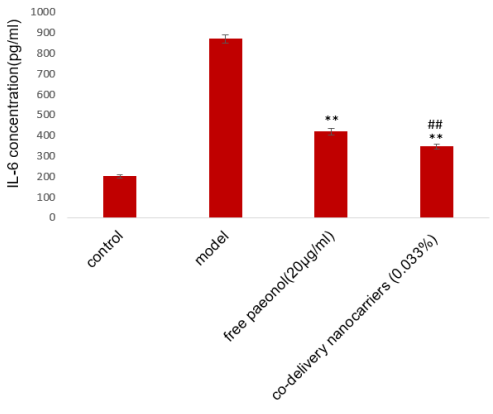

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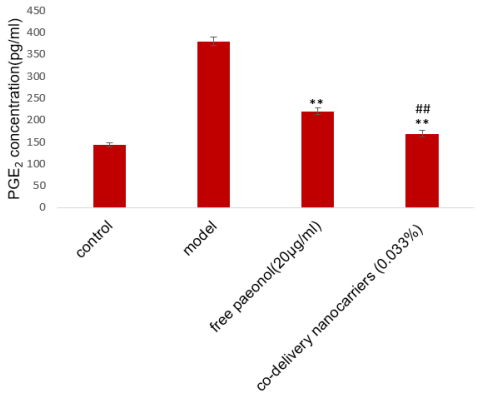
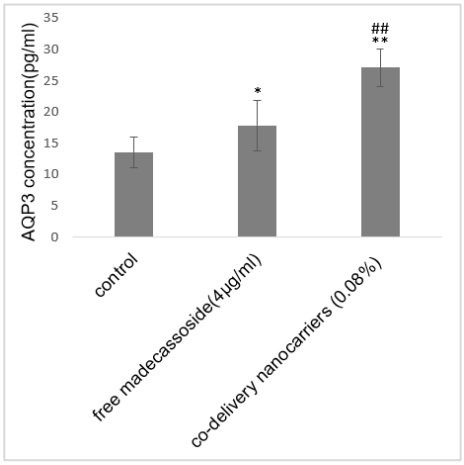


(c)

Figure 3. Effects of nanocarriers on LPS-induced inflammatory cytokine secretion in RAW264.7 macrophages. (a) IL-1α, (b) IL-6, and (c) PGE_2 levels. Data: mean ± SD; **p < 0.01, ***p < 0.001 vs. LPS model; #p < 0.05, ##p < 0.01 vs. free active compound.

3.5 Barrier Repair Effects

Aquaporin-3 (AQP3), a transmembrane protein facilitating small-molecule transport, is the most abundant aquaporin subtype in human skin. Filaggrin (FLG), a critical mediator of epidermal barrier function, regulates keratinocyte differentiation and stratum corneum formation. Claudin-1, a primary transmembrane protein in tight junctions (TJs), is essential for paracellular barrier integrit. The effects of nanocarriers (compared to free madecassoside at equivalent doses) on FLG, AQP3, and claudin-1 secretion in HaCaT cells are shown in Figure 4. As demonstrated in Figure 4a, nanocarriers significantly increased FLG secretion by 1.8-fold ($p < 0.05$) compared to free madecassoside. Figure 4b reveals that nanocarrier treatment elevated AQP3 levels by 2.1-fold ($p < 0.01$) relative to the free compound. Similarly, Figure 4c shows a 1.5-fold enhancement ($p < 0.05$) in claudin-1 secretion with nanocarriers. These results confirm the superior efficacy of nanocarriers in restoring skin barrier function compared to equivalent doses of free active ingredients.



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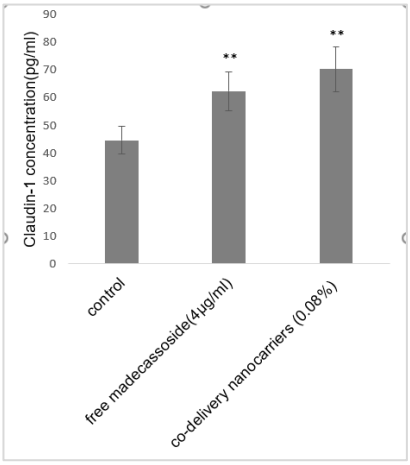

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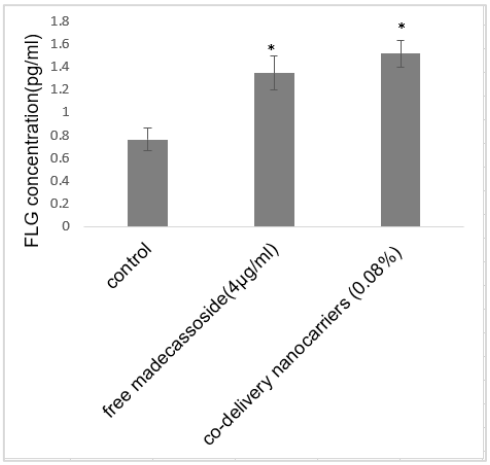

(c)

Figure 4. Effects of nanocarriers on barrier repair-related factors in HaCaT cells. (a) FLG secretion, (b) AQP3 expression, and (c) Claudin-1 levels. Data: mean ± SD; *p < 0.05, **p < 0.01 vs. control; #p < 0.05, ##p < 0.01 vs. free compound.

4. Discussion

The developed nanocarrier system co-delivering paeonol, madecassoside, and heparin sodium demonstrated significant enhancements in transdermal delivery and therapeutic efficacy. By achieving 116.3% and 124.5% increases in cumulative permeation and skin retention of paeonol, respectively, over free counterparts, the nanocarriers overcame bioavailability limitations through optimized size (~35 nm) and colloidal stability (PDI < 0.2). Confocal imaging confirmed rapid penetration into deeper dermal layers within 2 h, contrasting with free agents confined to the stratum corneum. Anti-inflammatory superiority was evidenced by reductions in IL-1α (48.2%), IL-6 (52.7%), and PGE_2 (41.9%) levels, likely due to synergistic interactions—paeonol's NF-κB suppression, heparin's chemokine neutralization, and madecassoside's COX-2 inhibition. Concurrently, the system elevated barrier repair markers (FLG: 1.8-fold; AQP3: 2.1-fold; claudin-1: 1.5-fold), addressing inflammation-barrier dysfunction interplay in dermatoses. While promising, scalability and in vivo validation remain critical for clinical translation.

5. Conclusion

This multifunctional nanocarrier system co-delivering anti-inflammatory and barrier-repairing actives addresses key limitations of conventional formulations by synergistically enhancing

transdermal delivery and therapeutic efficacy. Its dual capacity to reduce inflammatory mediators and upregulate barrier proteins positions it as a transformative platform for inflammatory dermatoses, where barrier dysfunction and immune dysregulation are intertwined. The observed synergy may stem from heparin-mediated stabilization of labile compounds and madecassoside-driven epidermal differentiation. With potential applications in premium "barrier-centric" skincare and medical formulations for chronic conditions like atopic dermatitis, this technology bridges cosmetic innovation and clinical dermatology. Future studies should prioritize in vivo validation of long-term safety and efficacy to unlock its full translational potential.

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