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Harnessing the Power of Hedyotis diffusa Extract: Molecularly Imprinted Iridoid Glycosides for Skin Soothing and Repair

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1. Introduction

Hedyotis diffusa Willd (*H. diffusa*), a perennial herb in the Rubiaceae family, is a renowned Chinese medicinal plant also known as *Oldenlandia diffusa Roxb*[1,2]. First documented in "Guangxi Traditional Chinese Medicine Annals", this herb has been traditionally valued for its heat-clearing, detoxifying, dampness-resolving, and jaundice-alleviating properties[3]. Clinically, it is widely used to treat furuncles, carbuncles, venomous snake bites, edema, and damp-heat jaundice, reflecting its substantial therapeutic potential[4,5]. The plant's bioactivity is attributed to key constituents such as iridoids (e.g., asperuloside, deacetylasperulosidic acid), flavonoids, and anthraquinones, which exert pharmacological effects by mitigating oxidative stress and regulating inflammatory pathways-properties highly relevant for skin-soothing and reparative cosmetic formulations[6,7].

However, translating *H. diffusa* extracts into safe and stable cosmeceuticals is impeded by two critical limitations: (1) the coexistence of irritant impurities (e.g., anthraquinones and specific terpenoids) that may trigger dermal irritation; and (2) the instability of iridoid glycosides under conventional extraction and storage conditions, which compromises active ingredient efficacy[8,9]. These challenges highlight the need for advanced separation technologies to enhance both purity and stability.

To address these bottlenecks, this study integrates molecular imprinting technology (MIT) with ultrasonic-assisted reflux extraction, leveraging MIT's ability to create synthetic receptor sites with specific binding affinity for target iridoid glycosides. By mimicking the recognition mechanism of antibodies or enzymes, MIT enables precise enrichment of bioactive compounds while excluding structurally similar impurities (e.g., methyl deacetylasperlosidate), thereby improving product purity. Ultrasonic-assisted reflux further enhances impurity removal through mechanical disruption and solvent penetration, optimizing the extraction efficiency of target components while minimizing residual irritants. This synergistic approach aims to overcome the limitations of traditional methods-such as complex compositions from solvent extraction or macroporous resin adsorption-and establish a targeted platform for isolating cyclohexene ether terpenoid glycosides with enhanced stability.

Herein, we report the development of a novel extraction process combining MIT and ultrasonic-assisted reflux to produce a *H. diffusa* extract with minimized irritant impurities and stabilized iridoid glycosides (Figure 1). Through systematic in vitro evaluations of its anti-oxidative, anti-inflammatory, and skin barrier-repairing mechanisms, as well as clinical assessments of safety and efficacy in sensitive skin populations, this study the integrated technology yields an extract with improved stability, reduced irritancy, and enhanced cosmeceutical performance. Success in this endeavor would not only address unmet needs in sensitive skin care but also establish a methodological paradigm for translating traditional herbal medicine into high-purity botanical extracts for modern cosmetic applications.

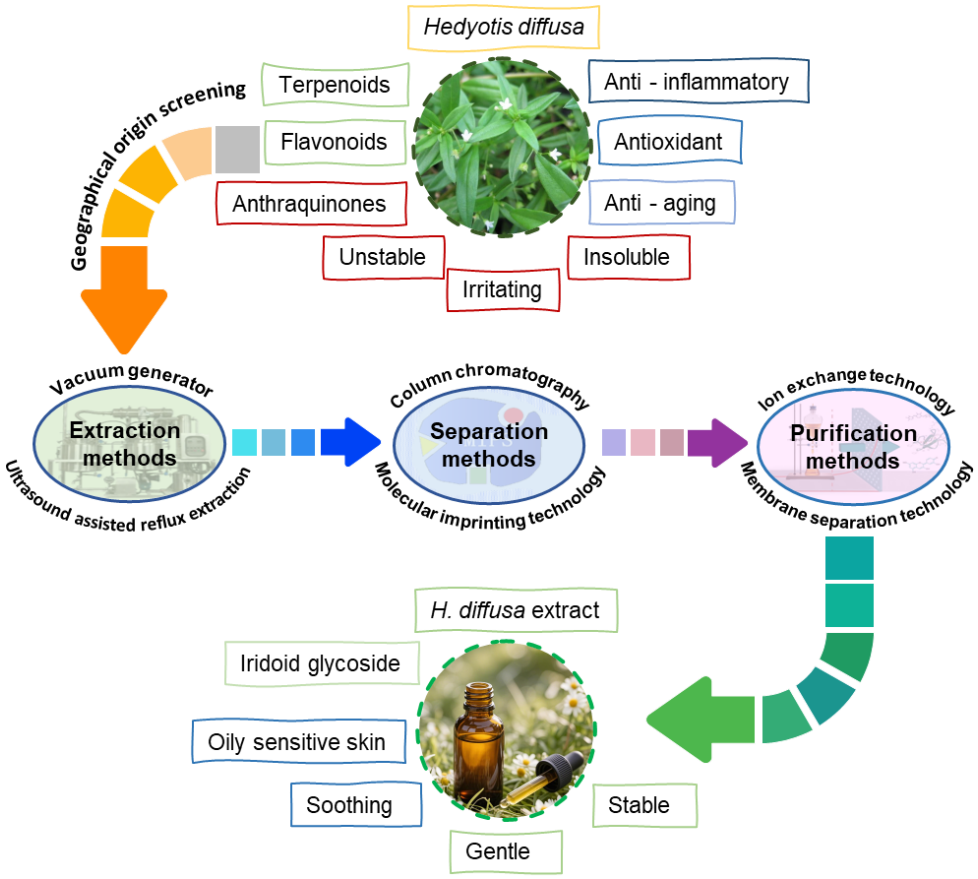

Figure 1. Schematic diagram of extraction, separation and purification of *H. diffusa*.

2. Materials and Methods

2.1 Preparation of *H. diffusa Extract*

H. diffusa Extract was obtained from Eneplus Biotechnology Co., Lt. Adopting ultrasound assisted reflux extraction method to improve extraction efficiency and reduce degradation of active ingredients; Simultaneously utilizing ion exchange technology, membrane separation technology, and molecular imprinting technology to selectively enrich and stabilize the bioactive component iridoid glycosides, removing impurities such as iridoid glycosides, anthraquinone, flavonoids, etc., thereby enhancing the stability and safety of the extract.

2.2 Content Stability evaluation

High-performance liquid chromatography (HPLC) with an external standard method was used to quantify components in crude and purified *H. diffusa* extracts. Samples (10 mg) were dissolved in 1 mL methanol, filtered through a 0.22 μm membrane, and analyzed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) equipped with a C18 column (4.6 × 250 mm, 5 μm). The mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B) under gradient elution: 5-15% A (0-10 min), 15-25% A (10-30 min), 25-35% A (30-40 min), at a flow rate of 1.0 mL/min. Detection was performed at 240 nm for iridoid glycosides (e.g., asperuloside, deacetylasperulosidic acid). Component stability was monitored over 12 weeks at room temperature and 48°C by comparing peak areas against external standards.

2.3 Mild and Stimulating Assessment

Chorioallantoic membrane (CAM) irritation testing followed the SN/T2329-2009 protocol. Fertilized chicken eggs were incubated at 37°C for 10 days, and the CAM was exposed. Samples (10% w/v degradation products of *H. diffusa* extract in PBS) and control compounds (oleuropein, gentiopicroside) were applied topically. After 48 hours of incubation at 48°C, irritation severity (hemorrhage, coagulation, vascular damage) was scored visually.

Zebrafish (Danio rerio) embryos (24 h post-fertilization) were exposed to 10% w/v extract degradation products or 0.1% SDS (positive control) in embryo medium. Stress-induced vibration frequency was recorded over 5 minutes using a stereomicroscope (Leica M205C), with PBS as the blank control.

2.4 Antioxidant and anti-inflammatory effects evaluation

Extract dilutions (0.8-4 mg/mL) were mixed with 0.1 mM DPPH methanol solution in 96-well plates and incubated in the dark for 30 minutes at 25°C. Absorbance at 517 nm was measured using a microplate reader (BioTek Synergy H1), and scavenging activity was calculated.

HaCaT keratinocytes (1×10^5 cells/well) were pretreated with 1% *H. diffusa* extract for 24 hours, followed by UVB irradiation (50 mJ/cm²). Cells were fixed, permeabilized, and incubated with anti-TRPV1 primary antibody (1:200, Abcam, UK), then Alexa Fluor 488-conjugated secondary antibody (1:400, Invitrogen). Nuclei were stained with DAPI, and fluorescence intensity was quantified *via* confocal microscopy (Leica TCS SP8).

HaCaT cells pretreated with the extract were stimulated with LPS (1 μg/mL) for 24 hours. Culture supernatants were collected, and IL-1α and PGE_2 levels were measured using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. Absorbance was read at 450 nm, and data were normalized to cell viability.

2.5 Skin barrier repair efficacy evaluation

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for H&E staining to visualize basal-layer cell apoptosis under light microscopy (Olympus BX53). For immunoflourence staining, sections of treated samples were incubated with anti-filaggrin (FLG, 1:100, Abcam) and anti-loricrin (LOR, 1:100, Santa Cruz Biotechnology) antibodies, followed by Alexa Fluor 594-conjugated secondary antibody (1:400). Nuclei were counterstained with DAPI, and fluorescence intensity was analyzed using ImageJ software, normalized to the blank control group.

2.6 Clinical study

Thirty-three female subjects (29-58 years, mean age 41.76 ± 8.23) with oily-sensitive skin (defined by TEWL > 25 $g \cdot h^{-1} \cdot m^{-2}$ and positive lactic acid stinging test) participated in a double-blind, randomized, self-controlled trial, applying a cream containing 1% *H. diffusa* extract twice daily for 28 days. Evaluations at baseline (Day 0) and Day 28 included objective measures transepidermal water loss (TEWL) *via* TM Hex probe, facial redness area *via* VISIA-CR® imaging], semi-objective assessment (lactic acid stinging test score), and subjective feedback (self-reported improvements in burning, stinging, redness, and tolerance on a 5-point scale). Statistical analyses used paired t-tests for within-group comparisons and ANOVA for between-group differences (placebo vs. extract), with significance set at $p < 0.05$.

2.7 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, USA). Student's t-test was used between two groups and ANOVA analysis was used for comparing multiple groups, with statistical significance set at $p < 0.05$.

3. Results

3.1 *H. diffusa* extract exhibits enhanced stability and reduced irritancy.

A comparison of HPLC chromatograms between extraction and purification products revealed that the *H. diffusa* extract obtained *via* the MIT process exhibited a significant reduction in irritating impurities[10]. Structurally similar compounds, such as deacetylasperulosidic acid and geniposidic acid, which contribute to irritation, were better-controlled in the purification products (Figure 2a). In a 12-week degradation experiment, the functional component asperuloside in *H. diffusa* extract showed remarkably enhanced stability, with a much lower degradation rate compared to other iridoid glycosides (e.g., oleuropein, asperuloside, gentiopicroside, swertiamarin) (Figure 2b). In the HET-CAM experiment, compared to other components (oleuropein, gentiopicroside, etc.), the *H. diffusa* extract exhibited minimal irritation severity after a 2-week accelerated degradation test at 48℃, and its degradation products did not induce significant irritation, highlighting its reduced irritancy profile (Figure 2c). In the zebrafish embryo experiment, unlike SDS, the *H. diffusa* extract did not induce stress-related vibration, further confirming its non-irritating property (Figure 2d). Collectively, these results demonstrate that the *H. diffusa* extract possesses enhanced stability and reduced irritancy.

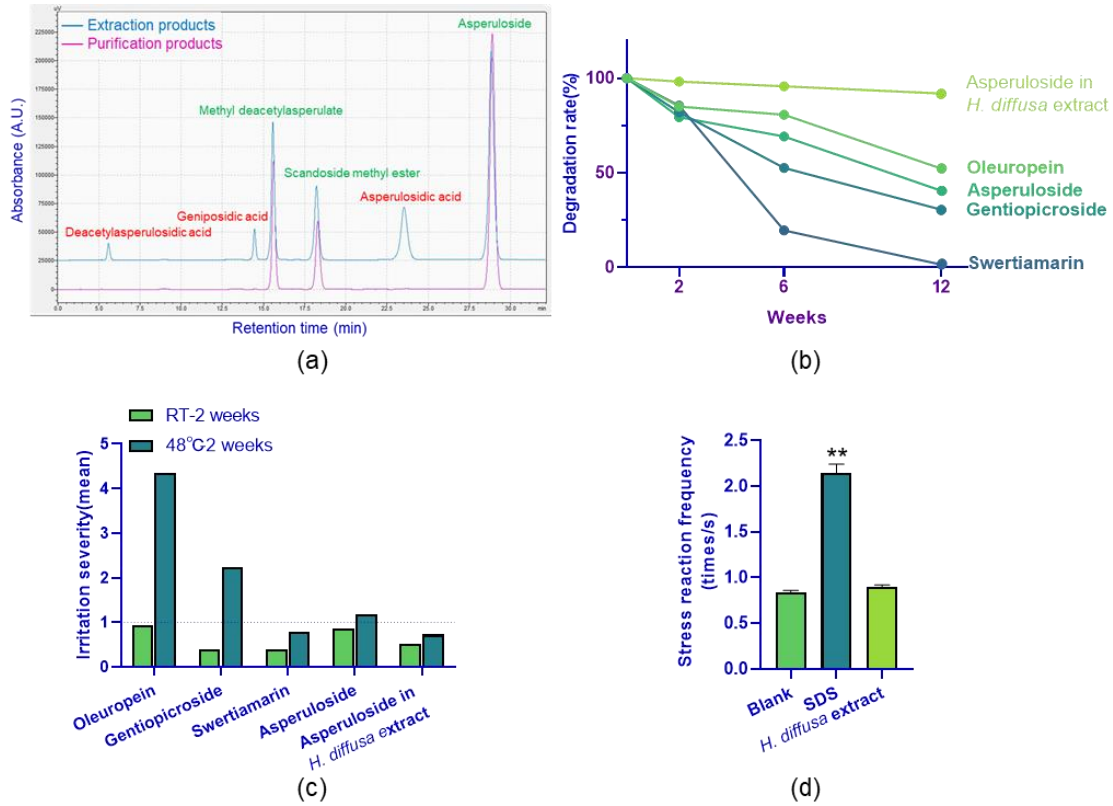

Figure 2. (a) HPLC chromatograms of extraction and purification products. (b) Degradation rate of components over 12 weeks. (c) Irritation severity in HET-CAM experiment. (d) Stress reaction in zebrafish embryo irritation evaluation.

3.2 *H. diffusa* extract displays potent anti-inflammatory and antioxidant activities

Sensitive skin is characterized by heightened reactivity, oxidative stress, and compromised barrier function, with overexpression of TRPV1, a receptor linked to pain and itch sensations, often exacerbating discomfort and inflammation[11]. The *H. diffusa* extract significantly reduced TRPV1 protein expression in keratinocytes (Figure 3a), suggesting potential to alleviate sensory hypersensitivity in sensitive skin.

Excessive nitric oxide (NO) production and pro-inflammatory cytokines like IL-1α and PGE_2 contribute to skin inflammation, manifesting as redness, swelling, and barrier damage. The *H. diffusa* extract effectively inhibited NO release, outperforming the positive control L-NMMA (Figure 3b), and suppressed LPS-induced inflammation by reducing IL-1α and PGE_2 levels (Figure 3c), demonstrating robust anti-inflammatory effects.

In antioxidant assays, the *H. diffusa* extract exhibited concentration-dependent DPPH scavenging activity (Figure 3d), indicating its capacity to counter oxidative stress-a critical factor in maintaining skin health and resilience. Collectively, these results highlight the *H. diffusa* extract's dual potency in mitigating inflammation and oxidative stress, positioning it as a promising ingredient for sensitive skin care formulations.

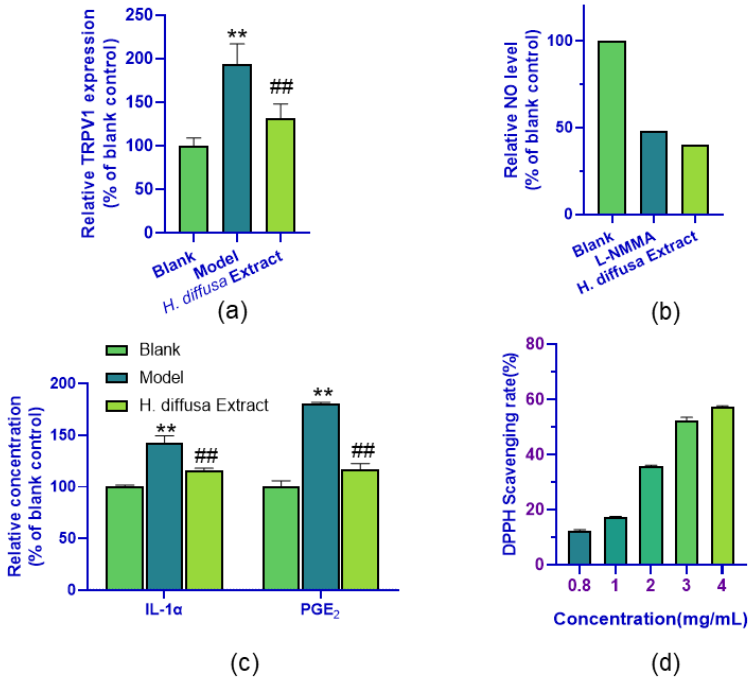

Figure 3. (a) Relative TRPV1 expression in keratinocytes. (b) Relative NO level. (c) Relative concentrations of *IL-1α* and PGE_2. (d) DPPH scavenging rate of *H. diffusa* extract at concentrations ranging from 0.8 to 4 mg/mL.

3.3 *H. diffusa* extract promotes skin barrier repair

Sensitive skin is often characterized by a compromised barrier function, rendering it susceptible to external stimuli such as sunburn and UV irradiation[12]. To evaluate the protective effect of *H. diffusa* extract on sensitive skin, we assessed its skin barrier repair efficiency in a UVB-treated reconstructed human epidermis model.

In the UVB irradiated reconstructed human epidermis model, UVB induced basal-layer epidermal cell shrinkage and apoptosis (Figure 4a). The *H. diffusa* extract effectively reduced the number of damaged cells caused by photothermal stress, demonstrating its protective effect on epidermal cells. The extract also restored the expression of FLG, a key barrier protein diminished by UVB (Figure 4b). Similarly, it increased the expression of LOR, an important protein for skin hydration and barrier integrity (Figure 4c). These results collectively indicate that the *H. diffusa* extract promotes skin barrier repair by safeguarding epidermal cells and restoring the expression of barrier-related proteins. This dual action enhances skin hydration and resistance to external damage, which is essential for preventing moisture loss and defending against environmental aggressors in sensitive skin care.

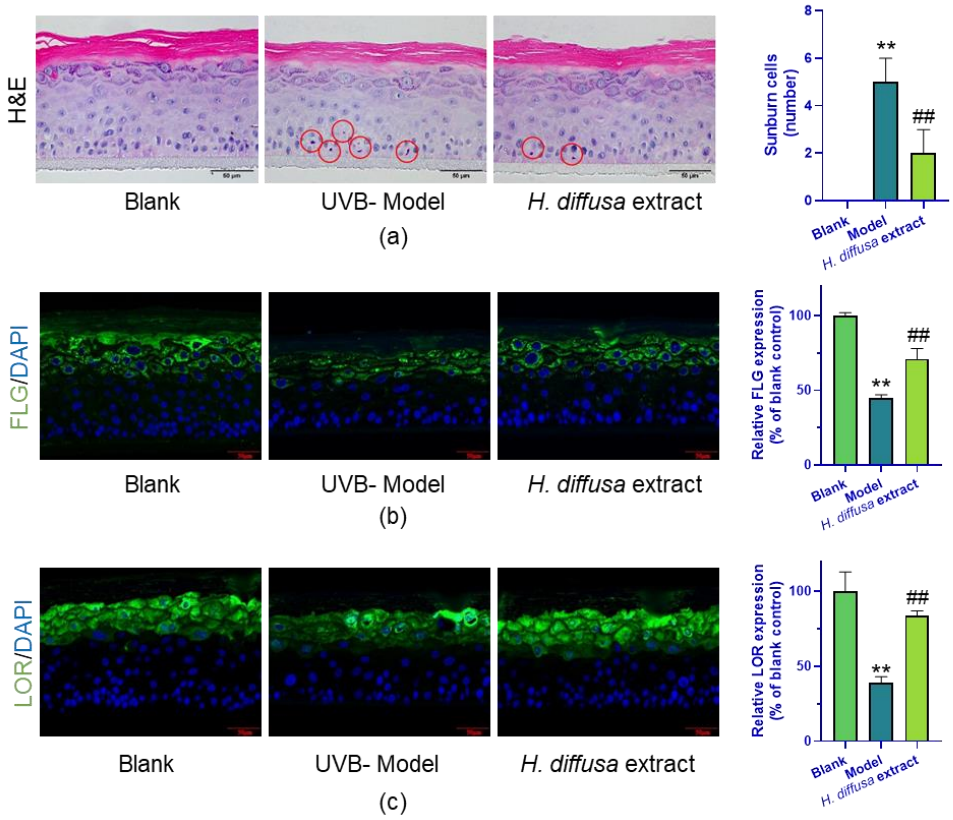

Figure 4. (a) H&E staining images (b) Immunofluorescence staining for filaggrin (FLG) expression. (c): Immunofluorescence staining for loricrin (LOR) expression. The quantitative analysis shows the relative LOR expression (as a percentage of the blank control). ** denotes a significant difference ($p < 0.01$) compared to the blank group; ## denotes a significant difference ($p < 0.01$) compared to the model group.

3.4 *H. diffusa* extract relieves objective symptoms of skin sensitivity

Skin sensitivity is typically marked by impaired barrier function, heightened susceptibility to stimuli, and visible erythema, profoundly affecting skin health and quality of life[13]. In volunteers with oily-sensitive skin, after a 28-day application of a formulation with 1% *H. diffusa* extract on facial skin, transepidermal water loss (TEWL) showed a significant improvement ($p < 0.01$), and the sensitive index in the lactic acid stinging test decreased (Figure 5a), indicating enhanced skin barrier integrity and reduced stimulus sensitivity. Questionnaire results indicated that over 90% of users reported the extract-containing product helped alleviate skin burning, stinging, and redness, while enhancing skin tolerance (Figure 5b), corroborating objective improvements. Following a 28-day application of 1% *H. diffusa* extract, VISIA analysis showed the *H. diffusa* extract group had a more substantial reduction in red-area percentage than the placebo, objectively reflecting alleviated skin erythema (Figure 5c and d). Collectively, these findings confirm *H. diffusa* extract effectively relieves objective symptoms of skin sensitivity, benefiting both barrier function and sensory comfort.

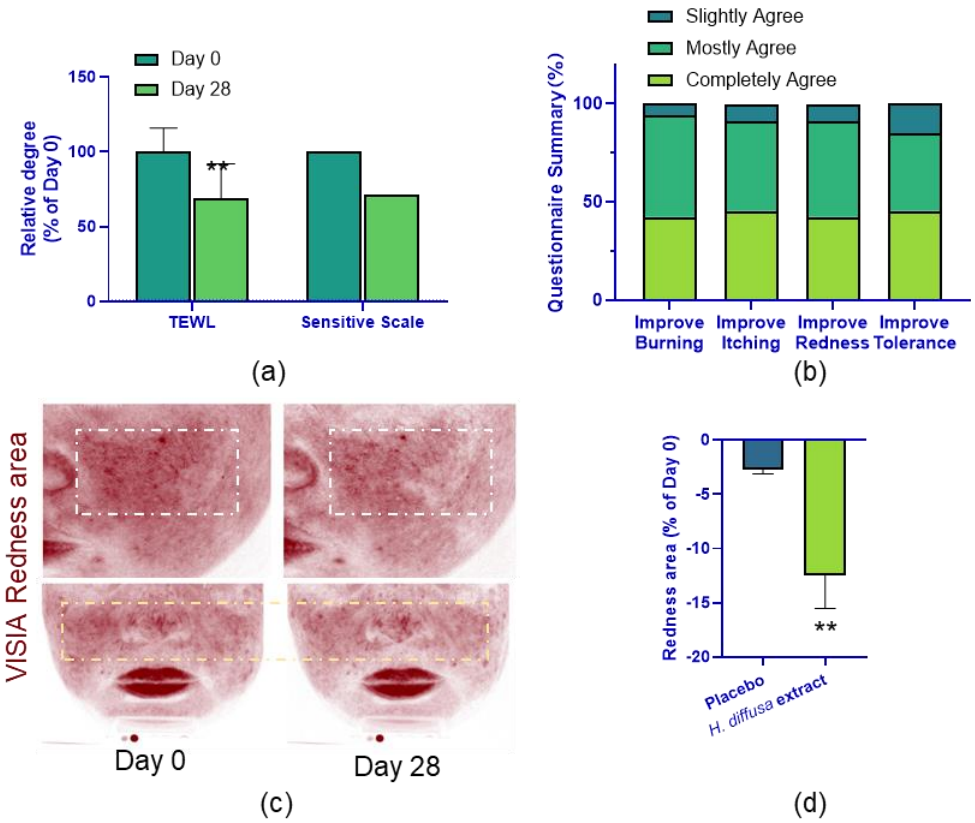

Figure 3. (a) Relative degree of TEWL (transepidermal water loss) and sensitive scale on Day 0 and Day 28. **Denotes a significant difference (p<0.01) between Day 0 and Day 28. **(b)** Questionnaire summary (%) on improvements in burning, itching, redness, and tolerance. **(c)** VISIA images of redness area on Day 0 and Day 28. The dashed-line boxes highlight the evaluated regions. **(d)** Percentage change in redness area. **Denotes a significant difference (p<0.01) between the placebo and *H. diffusa* extract groups.

4. Discussion

Our study presents a novel approach to isolate bioactive iridoid glycosides from *H. diffusa* using molecular imprinting technology (MIT) combined with ultrasonic-assisted extraction, yielding an extract with enhanced stability, reduced irritancy, and multifunctional benefits for sensitive skin. The core findings demonstrate that the integrated method selectively enriches target compounds while eliminating structurally similar irritants, addressing key limitations of traditional herbal extraction-instability and impurity-related irritation.

The scientific basis for these improvements lies in MIT's ability to mimic biological recognition, ensuring high-purity isolation of bioactive components like asperuloside. This not only stabilizes iridoid glycosides against degradation but also minimizes dermal irritation, as confirmed by in vitro models showing no induction of stress responses in zebrafish embryos or significant irritation in HET-CAM assays. Mechanistically, the extract exerts its effects through multiple pathways: downregulating TRPV1 expression in keratinocytes to mitigate sensory hypersensitivity, suppressing nitric oxide (NO) and pro-inflammatory cytokines (IL-1α, PGE_2) to reduce inflammatory responses, and restoring barrier proteins filaggrin (FLG) and loricrin (LOR) to

enhance epidermal integrity. These actions collectively address the core pathological features of sensitive skin-oxidative stress, inflammatory hyperreactivity, and barrier dysfunction[14].
The clinical validation further supports H. diffusa extract effect, showing improved transepidermal water loss (TEWL), reduced lactic acid sensitivity, and objective redness reduction in oily-sensitive skin volunteers. The high user satisfaction with alleviated burning, stinging, and redness indicates translational relevance, bridging in vitro mechanistic insights with real-world efficacy.
This study also establishes a methodological paradigm for modernizing traditional herbal medicine. By leveraging advanced separation technologies, it overcomes the inherent challenges of complex plant matrices, ensuring high-purity extracts with defined bioactivity. While focused on *H. diffusa*, the MIT-integrated approach is broadly applicable to other botanical sources, offering a blueprint for isolating unstable or impurity-prone phytochemicals. Future research could explore long-term safety profiles, synergistic formulations, and genetic expression profiling to further elucidate its molecular targets, enhancing its utility in precision dermatology.

5. Conclusion

Our study highlights a novel *H. diffusa* Extract，which provides active ingredients with mildness, stability, and strong efficacy. Through the specific process, the decomposition of active ingredients can be reduced, and the content of impurities can be controlled, providing a safe, mild, low stimulating, stable and excellent raw material for daily chemical applications with soothing, anti-inflammatory, repairing and other effects.

6. Conflict of Interest Statement.

NONE

7. References

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