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"Development of Extracellular Vesicles Loaded with Madecassoside for Enhanced Skin Penetration and Skin Conditions"

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1. Introduction

Extracellular vesicles (EVs), commonly referred to as exosomes, are lipid bilayer-enclosed particles naturally secreted by cells, carrying bioactive molecules such as proteins, lipids, RNA, and DNA. EVs play a key role in cell-to-cell communication by transferring their cargo to target cells, thereby modulating various physiological processes [1]. Recently, EVs have emerged as a promising material for diagnosing and treating diseases in medical applications. These biologically derived particles have garnered significant attention as promising carriers for bioactive molecule delivery due to their innate biocompatibility, stability, and ability to facilitate cellular communication [2]. Furthermore, due to their efficient cellular uptake and low toxicity compared to synthetic nanovesicles, EVs are increasingly recognized as natural nanocarriers for drug delivery and therapeutic applications.

Most cells are thought to release EVs, including some archaea, bacteria, fungi, and plants. Mammalian EVs hold substantial promise for therapeutic delivery; however, several obstacles impede their clinical application, such as the potential to stimulate cancer [3], rapid clearance from the bloodstream [4], and limited production yields [5]. In contrast, plant-derived extracellular vesicles (PDEVs) offer unique advantages such as scalability, low immunogenicity, and ethical acceptability, making them highly attractive for cosmetic and pharmaceutical applications [6]. PDEVs were found to have natural anticancer, anti-tumor, antioxidant, anti-inflammatory, and wound-healing effects, among many others, from an abundant variety of fruits and vegetables, each offering distinct and potent therapeutic effects [7]. They can efficiently encapsulate drugs while minimizing drug leakage and may serve as drug carriers that synergistically enhance the therapeutic effects of the drugs [8].

Madecassoside, a triterpenoid saponin isolated from *Centella asiatica*, is a well-known bioactive compound recognized for its potent anti-inflammatory, wound healing, and anti-aging properties [9]. However, due to its strong polarity, madecassoside has difficulty crossing the skin barrier and penetrating the skin, thus greatly hampering its use in skin care products [10]. Therefore, novel strategies to improve the delivery and stability of madecassoside are urgently needed to maximize its cosmetic and dermatological potential.

In this study, we aimed to develop a novel next-generation delivery system by encapsulating madecassoside into plant-derived EVs. We investigated the physicochemical properties, skin penetration ability, and bioactivity of the madecassoside-loaded EVs, with a view to their application as advanced cosmetic ingredients targeting skin health and rejuvenation.

2. Materials and Methods

Preparation of Apple Callus

Apple fruits (Malus domestica) for this experiment were sourced from the Apple Agricultural Cooperative Federation of Korea. The apples were washed thoroughly with running water and sterilized using 70% ethanol for 1 minute. A subsequent sterilization was performed with 1% sodium hypochlorite for 10 minutes, followed by washing three times with sterile distilled water. The sterilized fruit tissue was cut into pieces of 0.5 cm^2 and cultured in MS medium containing 4.5 mg/L 1-naphthylacetic acid (NAA) and 2.5 mg/L 6-Benzylaminopurine (BA) growth regulator. The pH of the medium was adjusted to 5.8, and the culture was maintained in a dark room at a 27 ± 3°C. In order to massive culture, 4 g of the apple callus were inoculated into an Erlenmeyer flask containing 50 mL of liquid medium with the same composition. The culture was incubated on a shaker (J-MBB2, JISICO, Republic of Korea) at 90 rpm for 3~4 weeks in the dark.

Preparation of Madecassoside-containing Apple EV

An isotonic solution was added to the mixture of apple callus and culture mediumand subsequently pulverized using a homogenizer (T50D, IKA, Aachen, Germany). Madecassoside was added and a pressure of 1,000 bar was applied using a high-pressure homogenizer (Microfludizer, MN600P-300, Picomax, Korea) three times to break down the cell walls and membranes via turgor pressure. Madecassoside was simultaneously loaded into EVs during separation process in culture media and plant cells. Cell wall debris was removed by centrifugation at 4°C 10,000×g, for 30 minutes. The supernatant was filtered through a 0.45 μm sterile filter following ultrafiltration (Pellicon® 2 and 3 Mini Holder, Merck, Germany) with a molecular weight cutoff (MWCO) of 100 kDa to separate and concentrate the EVs containing madecassoside. Impurities were additionally removed and sterilized by 0.2 μm filter. The isolated madecassoside-containing EVs (Madeca-EV) were diluted 200 times for use in subsequent tests. Nanoparticle Tracking analysis (NTA) were performed to verify the size of Madeca-EV and Madeca-EV samples using Nanosight NS300 (Malvern, UK).

Final sample for cell test were prepared with the following compositions: Madecassoside at 0.4 ppm; Madecassoside at 0.42 ppm with EVs (Madeca-EV) at 2.27×10^7 particles/mL; and Madecassoside at 4.53 ppm with EVs (Madeca-EV) at 1.80×10^7 particles/mL.

Cell culture

The immortalized human keratinocytes (HaCaT) and human fibroblasts (Hs68) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT) supplemented with 1% Antibiotic Antimycotic Solution and 10% fetal bovine serum in an atmosphere of 5% CO2 at 37℃.

Induction of inflammatory response

The HaCaT cells were maintained until 80% confluence and then cells were treated with Poly I:C 10 μg/ml + IL-4 10 ng/ml and each samplein serum-free medium and incubated for 4 hours. Dexamethasone (Dex) 1μM was used as positive controls.

Real-time RT-PCR

The total RNA from keratinocytes was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the recommended protocol. cDNA was synthesized from 2 μg of total RNA using a reverse transcription premix (Elpis-biotech, Daejeon, Korea) under the following reaction conditions: 45 °C for 45 min and 95 °C for 5 min. The signals of the gene expression were quantified with real-time polymerase chain reaction (PCR) amplification, and the data were analyzed using StepOne Plus software (Applied Biosystems, Waltham, MA). Real-time PCR amplification reactions were performed using an SYBR Green PCR Master Mix with premixed ROX (Applied Biosystems, Waltham, MA). The following primer pairs (Bioneer, Daejeon, Korea) were used in the reactions performed in an ABI 7300 following the manufacturer′s protocol: IL-1α (F: 5′-TGGCTCATTTTCCCTCAAAAGTTG-3′ and R: 5′-AGAAATCGTGAAATCCGAAGTCAAG-3′), IL-1β (F: 5′-GTCATTCGCTCCCACATTCT-3′ and R: 5′-ACTTCTTGCCCCCTTTGAAT-3′) and β-actin (F: 5′-GGCCATCTCTT-GCTCGAAGT-3′ and R: 5′-GACACCTTCAACACCCCAGC-3′).

Real-time PCR was performed using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Waltham, MA). The reaction conditions were as follows: initiation at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Expression of β-actin was used as an internal control.

Statistical analysis

The *in vitro* data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. All data were compared with a T-test. Statistical analyses were performed with a two-tailed Student's t-test. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Skin penetration of Madeca-EV

In order to analyze the penetration of Madeca-EV, a reconstructed full-thickness human skin model, KeraSkin™-FT (Biosolution Co., Ltd., Seoul, Korea) was used. KeraSkin™-FT was cultured on a 6-well plate containing tissue culture media (Welgene, Gyeongsang, Korea). The DiO-stained EVs were then applied to KeraSkin™-FT for 1 to 6 h. To visualize the skin permeabilized Madeca-EV, KeraSkin™-FT models were fixed in 4% formaldehyde (Sigma-Aldrich,

St. Louis, MO, USA) and embedded in paraffin. Subsequently, the tissue was cut vertically into 5-µm thick sections and attached to a glass slide (Paul marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). The tissues were counterstained by DAPI (VECTASHIELD; vector laboratories Inc., Burlingame, CA, USA) and observed under fluorescence microscopy (DM4000B; Leica, Wetzlar, Germany). For histological analysis, thin sections of the tissue were stained with hematoxylin (Merck KGaA, Darmstadt, Germany) and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA) and observed under a light microscope (Olympus Corporation, Tokyo, Japan).

3. Results

Isolation of Madecassoside-containing plant EVs

To investigate the potential of EVs as a next-generation bio-delivery system to advance cosmetic ingredients for better skin improvement, we generated plant EVs encapsulated madecassoside (Madeca-EV), a bioactive compound with regenerative properties, within these vesicles (**Figure. 1**).

Madecassoside introduced in the process of plant EV isolation from callus showed a concentration of 11.378mg immediately after EV isolation (**Table. 1**). Afterwards, when the madecassoside that was not introduced into the EV was removed through the washing process, it decreased to 5.818 mg. Through repeated washing process, the concentration of madecassoside gradually decreased, and finally, the concentration of madecassoside measured after washing 5 times was 0.004 mg. The number of particles of plant EVs separated through this process was 8.35×10^7. Finally, the amount of madecassoside contained in 10^{10} particles was 694 mg.

To evaluate the physicochemical properties of the madecassoside-loaded extracellular vesicles (Madeca-EVs), nanoparticle tracking analysis (NTA) was performed to determine the size distribution and concentration of the vesicles. The NTA results revealed that Madeca-EVs exhibited a relatively uniform size distribution with a main peak of particle diameter of approximately 124 nm (**Figure. 2**).

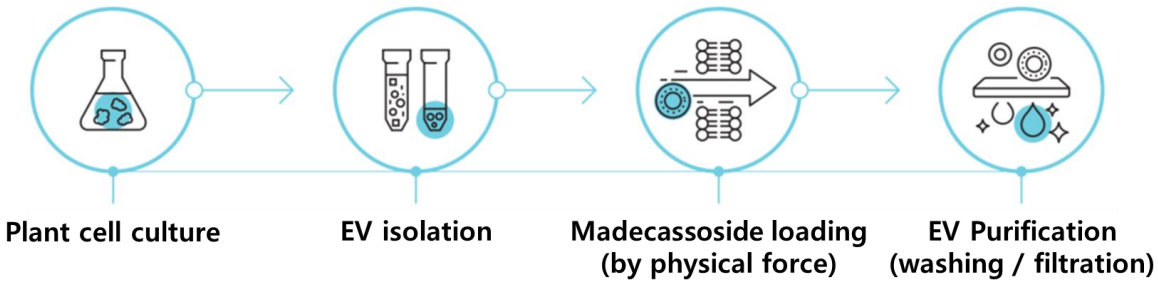

Figure 1. Schematic diagram of madecassoside loading into Apple EVs.

Process		Value
Madecassoside input (mg)		20,000
EV count (particles/ml)		8.35×10^7
Final madecassoside	Total (mg)	5.80
	In EV (mg/10^{10} particles)	694.61

Table 1. Madecassoside loading into Apple EVs (Madeca-EVs). Total 20 g of madecassoside was loaded into Apple EVs. After several washings, the amount of madecassoside loaded into EV was 694.61 mg.

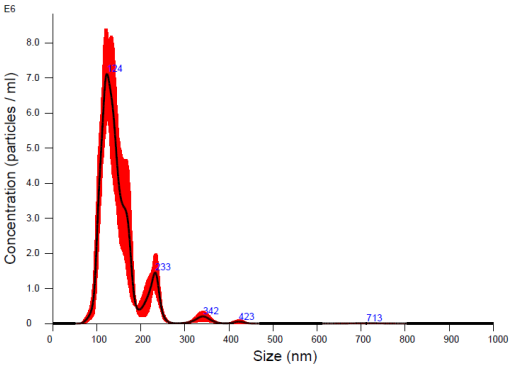

Figure 2. Nanoparticle Tracracking Analysis (NTA) data of Madeca-EV. Madecassoside loaded Madeca EV showed similar NTA size distribution with intact Apple EV.

Effect of Madeca-EV on the wound healing

To assess the enhanced biological efficacy of Madeca-EV on keratinocyte and fibroblast cells, in vitro cell-based assays were performed. Considering the well-documented role of madecassoside in promoting wound healing and reducing inflammation, we conducted scratch wound healing assays and measured inflammatory cytokine expression to evaluate the regenerative and anti-inflammatory potential of Madeca-EVs (**Figure. 3 & 4**). The wound healing effect of Madeca-EV was evaluated by migration assay using Hs68 fibroblast cells after cell scratch. In the scratch assay, Madeca-EV had skin regeneration capacity shown by cell migrating in Hs68 cells, compared with a non-treated control and positive control 1 μM Epigallocatechin gallate (EGCG) (**Figure. 3**). Treatment with Madeca-EVs significantly accelerated cell migration and wound closure compared to both the untreated control and cells treated with free madecassoside. Taken together, Madeca-EV suggests their potential as natural nutraceuticals to enhance skin health function. Further researches are necessary to elucidate their effects on skin health using clinical studies.

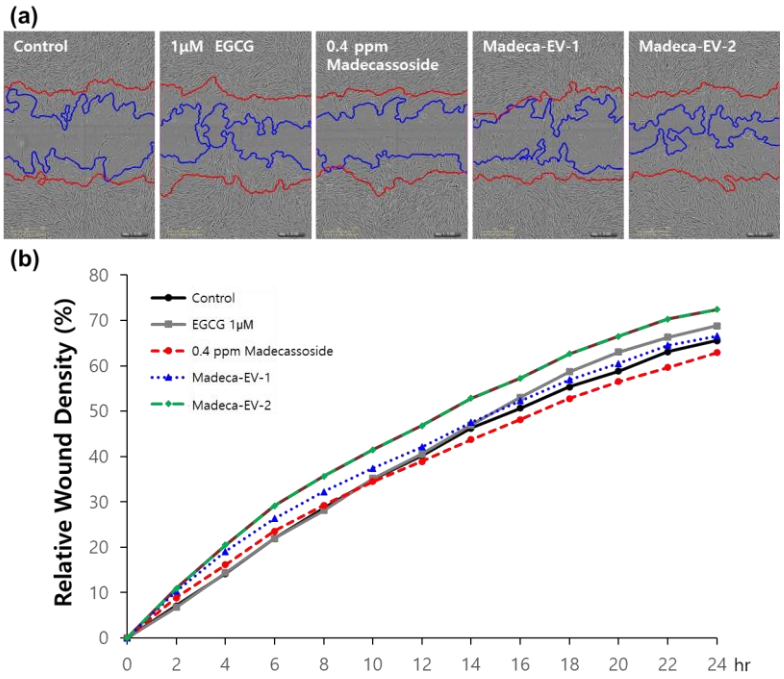

Figure 3. Wound healing capacity of Madca-EV. (a) Representative images of fibroblast cells scratch after 24 hr of treatment with each sample; (b) Relative wound confluence recovery results following sample treatment. 1μM EGCG was used as a positive control. Negative controls were performed using water. Madeca-EV-1 indicate 0.42 ppm madecassoside with EVs at 2.27 × 10^7 particles/mL and Madeca-EV-2 indicate 4.53 ppm madecassoside with EVs at 1.80 × 10^7 particles/mL.

Inhibitory effects of Madeca-EV on cytokine production

Furthermore, cytokine levels, interleukin-1 alpha (IL-1α) and beta (IL-1β), were evaluated after Madeca-EV treatment (**Figure 4**). Quantitative analysis of inflammatory markers revealed that Madeca-EV treatment more effectively suppressed the expression of IL-1α and IL-1β compared to free madecassoside treatment. As IL-1α and IL-6 are key cytokines involved in skin inflammation, these results demonstrate that Madeca-EV not only possesses anti-inflammatory effects but also contributes to formation of healthy skin barrier.

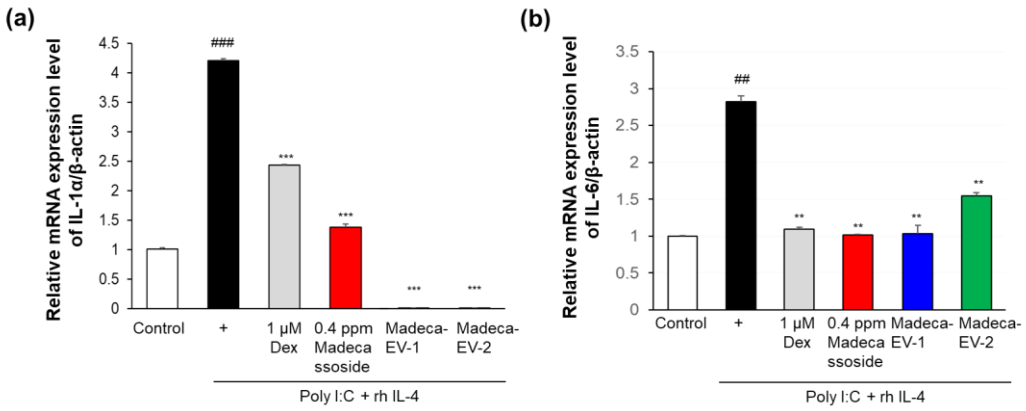

Figure 4. Relative gene expression of IL-1α (a) and β (b). HacaT were treated with each samples and Poly I:C (10 μg/ml) and IL-4 (10 ng/ml) to verify anti-inflammatory effects. 1 μM Dexamethasone (Dex) was used as a positive control.

Skin penetration of Madeca-EV

Using a human 3D skin model, we observed that DiO-labeled Madeca-EVs exhibited significantly increased fluorescence intensity, indicating enhanced skin penetration (**Figure 5**). This fluorescence were localized within the dermal layer of the skin. Notably, this finding suggest that Madeca-EVs facilitate the effective delivery and enhanced penetration of Madecassoside into the skin.

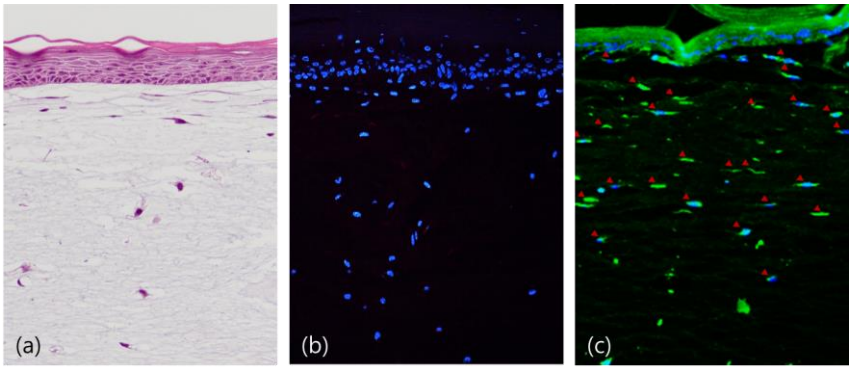

Figure 5. Skin penetration of Madeca-EV: (a) H&E staining of Keraskin™-FT, (b) DAPI staining of Keraskin™-FT, and (c) skin penetration of DiO-labeled Madeca-EV after 6 hours.

4. Discussion

In this study, we developed a novel delivery system by loading madecassoside into plant-derived EVs, termed Madeca-EVs, and evaluated their biological efficacy through *in vitro* assays. Consistent with previous reports demonstrating the regenerative and anti-inflammatory activities of madecassoside, our results showed that Madeca-EVs promoted wound healing and suppressed pro-inflammatory cytokine expression more effectively than free madecassoside in keratinocytes and fibroblasts (**Figure 3&4**). Although these findings are promising, further evaluations are necessary to comprehensively validate the efficacy of Madeca-EVs.

One important limitation of the current study is the lack of comparison with non-loaded (empty) EVs. Assessing the biological activities of EVs without madecassoside loading will help clarify the specific contribution of madecassoside encapsulation to the observed effects. This comparison is critical to determine whether the improved efficacy is solely due to madecassoside or if the EVs themselves possess intrinsic bioactivity.

Additionally, it is important to note that the current experiments were conducted in a conventional *in vitro* cell culture model using keratinocytes and fibroblasts. While the results from in vitro assays provided valuable insights, the inherent limitations of 2D cell culture systems must be acknowledged. In these simplified models, cells are directly exposed to treatment agents, potentially obscuring differences in delivery efficiency between free madecassoside and Madeca-EVs. Conversely, human skin is a multi-layered, three-dimensional structure comprising the stratum corneum, epidermis, and dermis. Each layer acts as a physical barrier that restricts molecular penetration, making the ability of Madeca-EVs to traverse these barriers a critical advantage that cannot be fully captured by two-dimensional *in vitro* models.

To extend the findings, experiments utilizing a 3D skin model were conducted, offering a closer approximation to the complexity of human skin compared to conventional *in vitro* cell culture models. . Future studies should investigate whether treatment with Madeca-EVs results in enhanced dermal penetration, and whether regenerative and anti-inflammatory markers are more significantly upregulated in 3D skin equivalents compared to treatment with free madecassoside or empty EVs alone. These additional evaluations will be essential to substantiate the application of Madeca-EVs as an advanced active ingredient for skin regeneration and inflammation control in cosmetic formulations.

Notably, madecassoside-loaded plant callus-derived exosomes showed significantly enhanced fluorescence intensity, indicating higher skin penetration, compared to madecassoside-loaded human stem cell-derived exosomes (data not shown). These findings suggest that EVs facilitate the effective delivery and enhanced penetration of madecassoside into the skin, further reinforcing the advantages of plant-derived EVs as a delivery platform.

The uptake process of EVs involves a complex three-step mechanism, including receptor interaction, membrane fusion, and subsequent internalization via endocytosis or phagocytosis [11]. This highly cell-specific process necessitates precise interactions between the surface molecules of recipient cells and EV proteins, enabling efficient targeting and adhesion. These characteristics underscore the importance of EVs in facilitating targeted delivery, a key feature demonstrated by Madeca-EVs.

We have developed a new regenerative cosmetic ingredient that utilizes this system to enhance the effects of Madecassoside and improve its penetration into the skin, offering promising applications in skincare formulations.

5. Conclusion

In this study, we investigated the potential of EVs as a next-generation bio-delivery system to advance cosmetic ingredients for better skin improvement. We isolated EVs from plant and encapsulated madecassoside, a bioactive compound with regenerative properties, resulting in Madeca-EV. Madeca-EVs exhibited enhanced effects on fibroblast proliferation, indicating improved cellular regeneration compared to free madecassoside. Additionally, these materials significantly reduced the expression of inflammation-related markers, such as IL-1α, suggesting anti-inflammatory properties.

Importantly, we observed that DiO-labeled Madeca-EVs effectively penetrated and localized within the dermal layer of 3D skin. These findings suggest that EVs facilitate the effective delivery and enhanced penetration of madecassoside into the skin.

To summarize, this study provides evidence that plant-derived extracellular vesicles (EVs) can serve as an effective alternative to human-derived exosomes, underscoring their potential as efficient drug delivery systems. Due to their inherent biological activity, plant-derived EVs not only act as delivery vehicles but also offer synergistic effects with the encapsulated substances. Through this study, we have developed a novel regenerative cosmetic ingredient that harnesses this system to enhance the effects of madecassoside and improve its penetration into the skin, suggesting promising applications for next-generation delivery system in skincare formulations.

6. Reference

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