

Article

Taiwanofungus camphoratus-Derived Extracellular Vesicles as a Potential Cosmetic Bioactive Candidate for Protecting Keratinocytes from UVB-Induced Photoaging

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Abstract

Ultraviolet B (UVB)-induced photoaging is characterized by oxidative stress, mitochondrial dysfunction, and epidermal barrier impairment. Although extracellular vesicles (EVs) have emerged as bioactive mediators of intercellular signaling, the dermatological relevance of fungal-derived EVs remains poorly understood. In this study, we investigated whether EVs isolated from liquid-cultured *Taiwanofungus camphoratus* mycelia (TCEVs) protect keratinocytes against UVB-induced damage. TCEVs displayed nanosized vesicular features with a mean particle size of 134.6 ± 4.6 nm and a stable negative surface charge. In HaCaT cells, TCEVs showed no obvious cytotoxicity and increased wound closure. In UVB-exposed keratinocytes, TCEV pretreatment improved cell viability, increased ATP production, reduced intracellular reactive oxygen species accumulation, and improved mitochondrial membrane potential. At the highest tested concentration (10×10^{10} particles/mL), TCEVs reduced UVB-induced relative ROS levels from 118.65% to 98.2% and increased relative ATP levels from 70.47% to 86.0%. TCEVs also increased the expression of ZO-1, occludin, and Claudin-3 and were associated with increased SIRT1 and p-AMPK expression and partial improvement of PGC-1 α . In contrast, Nrf2 and TFAM did not differ significantly from the UVB-irradiated group. These findings support the potential of TCEVs to prevent UVB-induced keratinocyte damage under the present in vitro conditions.

Keywords: *Taiwanofungus camphoratus*; extracellular vesicles; cosmetics; UVB; photoaging; keratinocytes; mitochondrial function; oxidative stress



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1. Introduction

The skin is the first biological barrier protecting the human body against external environmental insults, and its barrier function is primarily maintained by the stratum corneum and intercellular tight junctions (TJs). Tight junction proteins, such as ZO-1, occludin, and members of the claudin family, regulate paracellular permeability between epidermal cells and are closely associated with overall skin barrier stability and transepidermal water loss (TEWL) [1–4]. Among extrinsic aging factors, ultraviolet radiation, particularly UVB, is considered one of the major contributors to skin photoaging, as it induces oxidative stress, disrupts cellular signaling and barrier homeostasis, and ultimately leads to structural and functional deterioration of the skin [5–7].

At the cellular level, mitochondrial dysfunction is regarded as a critical driving mechanism of UVB-induced photoaging. In addition to ATP production, mitochondria are key hubs for the metabolism of reactive oxygen species (ROS) and for cellular stress responses. Recent reviews have indicated that when skin cells are exposed to UV or other external stressors, mitochondrial dynamics, energy metabolism, and quality-control systems become dysregulated, thereby amplifying oxidative damage, inflammatory responses, and aging phenotypes [8–11]. Furthermore, UV-induced mitochondrial disturbances and loss of membrane potential have been directly observed in human epidermal keratinocytes, suggesting that maintaining mitochondrial homeostasis is an important strategy for delaying skin aging and promoting repair [12].

In recent years, extracellular vesicles (EVs) have attracted considerable attention because they possess a lipid bilayer structure, can carry proteins, lipids, nucleic acids, and metabolites, and mediate intercellular communication [13,14]. Among naturally derived EVs, plant-derived EVs have been regarded as promising candidates with scalable production potential [15,16]. Fungal-derived EVs are naturally secreted membrane-bound particles that can transport proteins, lipids, carbohydrates, nucleic acids, and other molecules and may participate in intercellular and cross-kingdom communication [17–20]. Compared with intact mycelia or crude fungal extracts, an isolated EV preparation permits direct evaluation of the secreted nanoscale fraction, reduces direct exposure of HaCaT cells to intact fungal structures and bulk culture-medium components, and allows in vitro doses to be standardized by particle number. These features provide a rationale for studying TCEVs as a distinct material, although they do not establish that TCEVs are inherently safer or more effective than whole fungi or crude extracts.

Taiwanofungus camphoratus is a medicinal fungus endemic to Taiwan and has been reported to possess multiple bioactivities, including antioxidant, anti-inflammatory, immunomodulatory, and metabolic regulatory effects [21–23]. Compared with naturally grown or wood-cultivated fruiting bodies, mycelia can be produced through controlled liquid fermentation, supporting raw material consistency, standardization, and scale-up [22,24,25]. *Taiwanofungus camphoratus* mycelia and culture filtrates have also shown antioxidant and immunomodulatory activities [26–28]. From a cosmetic-development perspective in Taiwan, candidate ingredients require quality, safety, and functional documentation under applicable Taiwan Food and Drug Administration requirements. Existing toxicological information for *Taiwanofungus camphoratus* mycelia, fruiting bodies, or extracts cannot be directly extrapolated to isolated TCEVs.

Previous *Taiwanofungus camphoratus* studies have mainly focused on mycelia, fruiting bodies, culture filtrates, or their extracts, whereas the effects of *Taiwanofungus camphoratus*-derived extracellular vesicles on UVB-induced oxidative stress, mitochondrial dysfunction, and barrier-related protein changes remain insufficiently characterized. Therefore, this study aimed to isolate and characterize TCEVs and to evaluate their effects on UVB-induced oxidative stress, mitochondrial function, and epidermal barrier-related proteins in HaCaT keratinocytes. A pretreatment design was used to examine preventive and cytoprotective effects rather than post-injury regeneration. By integrating these outcomes in a single UVB keratinocyte model, this study addresses previously limited evidence concerning fungal-derived EVs in skin photodamage.

2. Materials and Methods

2.1. Reagents and Materials

High-glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA; 12100-046), fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 mM sodium pyruvate (Corning, Corning, NY, USA; 25-000-CI), penicillin–streptomycin (Gibco, USA;

15140-122), ATP detection assay kit-luminescence (Cayman Chemical, Ann Arbor, MI, USA), DMSO (Sigma-Aldrich, Darmstadt, Germany; D2650), Enhanced Cell Counting Kit-8 (Elabscience, Wuhan, China; E-CK-A362), Pierce™ RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA), protease inhibitor cocktail (GoalBio, Taipei, Taiwan; HC100-007), phosphatase inhibitor cocktail (GoalBio, Taiwan; HC100-008), bovine serum albumin (Gibco, USA), Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA), 5× protein sample dye (GeneMark, Taichung, Taiwan; GM47-b), mitochondrial membrane potential assay kit (JC-1; Abbkine, Wuhan, China; KTA4001), resveratrol ($\geq 99\%$ HPLC; Sigma-Aldrich, Germany; R5010), and H₂DCFDA (2',7'-dichlorofluorescein diacetate; Sigma-Aldrich, Germany; D6883).

2.2. Preparation of *Taiwanofungus camphoratus* Mycelial Culture Broth

The *Taiwanofungus camphoratus* strain used in this study was isolated and maintained by the Experimental Forest, College of Bio-Resources and Agriculture, National Taiwan University, Taipei, Taiwan. Molecular identification was performed by NCBI BLASTN analysis of a 642 bp sequence, which showed 100% query coverage and 100% sequence identity with reference *Taiwanofungus camphoratus* sequences (E-value = 0.0). The mycelia were cultured in potato dextrose broth (PDB) under liquid fermentation conditions at 25 °C and 200 rpm for 7–14 days. After cultivation, the mycelial culture supernatant was collected as the starting material for TCEV isolation.

2.3. Isolation and Preparation of TCEVs

Each TCEV batch was prepared from 60 mL of *Taiwanofungus camphoratus* mycelial culture supernatant by differential centrifugation at 4 °C. The starting material was centrifuged at 3000× g for 30 min to remove fungal cells and large particles, followed by 10,000× g for 30 min to remove cell debris and larger impurities. The clarified supernatant was then centrifuged at 100,000× g for 2 h at 4 °C using an Optima L-90K ultracentrifuge (Beckman Coulter, Brea, CA, USA). The resulting pellet was resuspended in 1 mL of sterile PBS. The final TCEV preparation contained $(2.33 \pm 2.01) \times 10^{13}$ particles/mL, corresponding to a calculated yield of approximately $(3.88 \pm 3.35) \times 10^{11}$ particles per milliliter of original culture supernatant. This value represents a volume-normalized particle yield rather than a recovery rate because the particle number before isolation was not measured. The resulting TCEV suspension was stored at −80 °C and used directly for physico-chemical characterization and cell-based experiments without any additional nanoparticle synthesis procedure.

2.4. Nanoparticle Tracking Analysis (NTA)

To determine the size distribution and particle concentration of TCEVs, nanoparticle tracking analysis was performed at the Instrumentation Center of Kaohsiung Medical University using a ZetaView® particle analyzer (Particle Metrix GmbH, Inning am Ammersee, Germany). Before analysis, the TCEV suspension was diluted 1000-fold in PBS. Samples were measured at 25.3 °C, and videos were recorded at 11 predefined positions, with each position scanned three times. The camera sensitivity was set to 85, and the shutter speed was fixed at 100. Particle size distribution and concentration were recorded as physical characteristics of the TCEV preparation. Particle concentrations determined by NTA were used to standardize all TCEV treatment doses, which were expressed as particles/mL; total EV-associated protein was not used for dose normalization.

2.5. Particle Size Distribution and Zeta Potential Analysis

The particle size homogeneity and surface charge of TCEVs were measured by dynamic light scattering (DLS) and electrophoretic light scattering using a Zetasizer Nano

ZS90 (Malvern Instruments, Malvern, Worcestershire, UK). For DLS measurements, the detection angle was set at 90°, the measurement temperature was 25 °C, and the viscosity and refractive index of the dispersant were set to 0.8872 mPa·s and 1.330, respectively. Samples were resuspended in double-distilled water to approximately 0.5–1 mL and loaded into DTD0012 (Malvern Instruments, Worcestershire, UK) disposable cuvettes for particle size analysis. Zeta potential analysis was conducted at 25 °C using DTS1070 (Malvern Instruments, Worcestershire, UK) folded capillary cells. The dielectric constant and viscosity of the dispersant (water) were set to 78.5 and 0.8872 mPa·s, respectively, and the zeta potential was calculated from electrophoretic mobility using the Smoluchowski model. All measurements were performed in triplicate, and the average values were reported.

2.6. Transmission Electron Microscopy (TEM) Observation

To observe the morphology of TCEVs, an aliquot of the ultracentrifugation-derived TCEV suspension was further concentrated solely for TEM preparation using a 100 kDa molecular weight cut-off (MWCO) membrane at 1000× *g* for 10 min at 4 °C until a final volume of 100 µL was obtained. This additional MWCO concentration step was not applied to samples used for NTA, DLS, zeta-potential measurements, or cell-based assays. A 10 µL aliquot was applied to a copper grid for 20 min, fixed with 10 µL of 1% paraformaldehyde for 15 min, negatively stained with 10 µL of 1% (*w/v*) uranyl acetate, rinsed with double-distilled water, air-dried, and left overnight. TEM observation was performed using a JEM-1400 Flash transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV with technical assistance from the Instrumentation Center of National Chung Hsing University.

2.7. Cell Culture

HaCaT cells, an immortalized human keratinocyte cell line, were kindly provided by Dr. Lai at National Chung Hsing University. The cell line, CVCL_0038, was obtained from AddexBio (Catalog # T0020001, San Diego, CA, USA). Cells were cultured in high-glucose DMEM (Gibco, USA; 12100-046) supplemented with 10% (*v/v*) fetal bovine serum, 1% (*v/v*) penicillin–streptomycin (Gibco, USA; 15140-122), and 3.7 g/L sodium bicarbonate (NaHCO₃), and maintained in a humidified incubator at 37 °C with 5% CO₂. Cells at appropriate confluence were used for subsequent functional assays.

2.8. Effects of TCEVs on HaCaT Cell Viability

To evaluate the cytotoxicity of TCEVs, HaCaT cells were seeded at a density of 2×10^4 cells/well in 96-well plates and incubated for 24 h. Cells were then treated with different concentrations of TCEVs (0.625, 1.25, 2.5, 5, and 10×10^{10} particles/mL) for 24 h, while resveratrol (25 µM) served as the positive control. After treatment, the culture medium was replaced with fresh medium containing 1% (*v/v*) Enhanced Cell Counting Kit-8 reagent and incubated at 37 °C for 1 h. Absorbance was measured at 450 nm using a microplate spectrophotometer (µQuant, BioTek, Winooski, VT, USA). Cell viability was calculated as follows: cell viability (%) = (OD₄₅₀ of treatment/OD₄₅₀ of control) × 100.

2.9. Cell Scratch Assay

To evaluate the effects of TCEVs on HaCaT cell migration, cells were seeded at a density of 1×10^6 cells/well in 6-well plates and incubated for 24 h until a complete monolayer formed. A straight scratch was made across the cell monolayer using a sterile pipette tip. Detached cells and debris were removed, and cells were subsequently treated with TCEVs (2.5, 5, and 10×10^{10} particles/mL) or resveratrol (25 µM). Images of the wound area were captured at 0, 12, and 24 h using an upright fluorescence microscope (ECLIPSE Ci, Nikon, Tokyo, Japan), and wound closure (%) was quantified using ImageJ software (version 1.54t; National Institutes of Health, Bethesda, MD, USA).

2.10. UVB Irradiation and TCEVs Treatment Procedure

HaCaT cells were seeded at a density of 2×10^4 cells/well in 96-well plates and incubated for 24 h. To establish a preventive/cytoprotective model, cells were pretreated with TCEVs or resveratrol for 6 h before UVB exposure. To maintain the same vehicle condition across treatment groups, the TCEV-treated and resveratrol groups were maintained in culture medium containing 0.1% DMSO during pretreatment. Cells were then washed with $1 \times$ PBS and irradiated in PBS using a UVP CL-3000 UV CrossLinker (Analytik Jena, Jena, Germany) equipped with UVB lamps with a peak wavelength of 302 nm. The culture plate was placed horizontally 15 cm below the lamps so that all wells were positioned on the same irradiation plane. A target dose of 150 mJ/cm^2 was entered into the instrument, which automatically calculated the exposure duration according to the current lamp output and stopped irradiation when the preset dose was reached. Under the present conditions, the exposure time was approximately 26 s. After irradiation, PBS was immediately replaced with fresh culture medium, and cells were incubated for an additional 24 h before analysis. TCEVs were tested at 2.5, 5, and 10×10^{10} particles/mL, and resveratrol (25 μM) served as an independent positive control.

2.11. Intracellular Reactive Oxygen Species (ROS) Assay

Intracellular ROS accumulation was determined using fluorescent probe H_2DCFDA . After treatment, the culture medium was removed, and cells were washed twice with $1 \times$ PBS. H_2DCFDA working solution prepared in PBS at a final concentration of 30 μM was then added, and the cells were incubated at 37 °C in the dark for 30 min. Fluorescence intensity was measured using a multilabel microplate reader (Hidex, Turku, Finland) at excitation/emission wavelengths of 485/535 nm. ROS production was expressed as a percentage relative to the control group and calculated using the following formula: ROS production (%) = (Treatment $A_{485/535}$ /Control $A_{485/535}$) $\times$ 100.

2.12. Intracellular ATP Assay

Intracellular ATP content was measured using a luminescence-based ATP assay. Reactions were performed in accordance with the ATP detection assay kit-luminescence protocol (Cayman Chemical, USA), and luminescence signals were detected using a multilabel microplate reader (Hidex, Finland). ATP concentrations were determined using a standard curve and expressed as a percentage of the control group to evaluate the ability of TCEVs to ameliorate UVB-induced metabolic dysfunction.

2.13. Mitochondrial Membrane Potential Analysis ($\Delta\psi_m$)

Mitochondrial membrane potential was evaluated using JC-1 staining. After treatment, the cells were stained according to the instructions provided in the JC-1 mitochondrial membrane potential assay kit (Abbkine, China; KTA4001). Red aggregate fluorescence and green monomer fluorescence were observed using an upright fluorescence microscope (ECLIPSE Ci, Nikon, Japan) equipped with a 40 $\times$ objective lens. Images were captured using a microscopic imaging system (SGHD-3.6, SAGE Vision, New Taipei City, Taiwan) connected to SG Image V2.3 software (Taiwan), and fluorescence quantification and channel merging were performed using ImageJ software (National Institutes of Health, USA). The red/green fluorescence ratio was used as an indicator of mitochondrial membrane potential to assess UVB-induced mitochondrial dysfunction and the protective effects of TCEVs.

2.14. Western Blot Analysis

After treatment, the cells were washed with ice-cold PBS and lysed with Pierce™ RIPA buffer (Thermo Fisher Scientific, USA) supplemented with a 1% protease inhibitor

cocktail (GoalBio, Taiwan; HC100-007) and phosphatase inhibitor cocktail (GoalBio, Taiwan; HC100-008). Lysates were incubated on ice with intermittent vortexing, centrifuged at high speed, and the supernatants were collected and stored at -80°C . The protein concentration was determined using Bio-Rad protein assay dye reagent concentrate (Bio-Rad, USA). After adjusting to a uniform concentration, the samples were mixed with $5\times$ protein sample dye (GeneMark, Taiwan; GM47-b) and denatured at 37°C for 5 min. The proteins were separated on a 12% SDS-PAGE and transferred to PVDF membranes (Revvity, Waltham, MA, USA) at 300 mA for 1 h. Membranes were washed with TBST, blocked with EZBlocker (GenePure, Taichung, Taiwan) at room temperature for 90 min, and then incubated overnight at 4°C with primary antibodies against ZO-1 (1:10,000; 21773-1-AP, Proteintech, Wuhan, China), occludin (1:10,000; 27260-1-AP, Proteintech, China), TFAM (1:20,000; 22586-1-AP, Proteintech, China), PGC-1 α (1:10,000; 66369-1-Ig, Proteintech, China), Claudin-3 (1:1000; 34-1700, Invitrogen, Carlsbad, CA, USA), AMPK α 1 (1:1000; 07-350, Millipore Sigma, Burlington, MA, USA), p-AMPK α (1:1000; 2535, Cell Signaling Technology, Danvers, MA, USA), SIRT1 (1:1000; 9475S, Cell Signaling Technology, USA), Nrf2 (1:1000; 12721, Cell Signaling Technology, USA), and β -actin (1:5000; SC-47778, Santa Cruz, Dallas, TX, USA). After primary antibody incubation, the membranes were washed with TBST and incubated with secondary antibodies, including HRP-conjugated goat anti-mouse IgG (1:10,000; ARG65350, Arigo Biolaboratories, Hsinchu, Taiwan) or HRP-conjugated goat anti-rabbit IgG (1:10,000; abs-22-200, Asia Bioscience, Taipei, Taiwan), at room temperature for 2 h. Immunoreactive bands were visualized using WesternBrightTM ECL substrate (Advansta, San Jose, CA, USA), and β -actin was used as the internal control for semi-quantitative analysis.

2.15. Statistical Analysis

All experiments were performed at least three times independently. Data are presented as mean $\pm$ standard deviation (SD), and the n values for each experiment are indicated in the corresponding figure legends. Statistical analyses were performed using GraphPad Prism 8.0 software (Dotmatics, San Diego, CA, USA). Differences among multiple groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Physicochemical Characterization and Morphological Observation of TCEVs

The overall TCEV isolation procedure is schematically illustrated in Figure 1A, including sequential centrifugation at $3000\times g$ and $10,000\times g$ followed by ultracentrifugation at $100,000\times g$ and PBS resuspension. NTA and TEM were used as complementary characterization methods in accordance with the principle of multimethod EV characterization described in MISEV2023 [29]. NTA showed a mean particle size of 134.6 ± 4.6 nm and provided the particle-size distribution and concentration of the preparation (Figure 1B). The polydispersity index was 0.162 ± 0.009 , and the zeta potential was -19.4 ± 0.43 mV (Figure 1C).

TEM images showed representative spherical or cup-shaped membrane-bound particles (Figure 1D). Together, the NTA and TEM findings support the presence of nanosized vesicular particles in the TCEV preparation; these methods provide complementary physical characterization evidence but do not independently establish absolute EV purity.

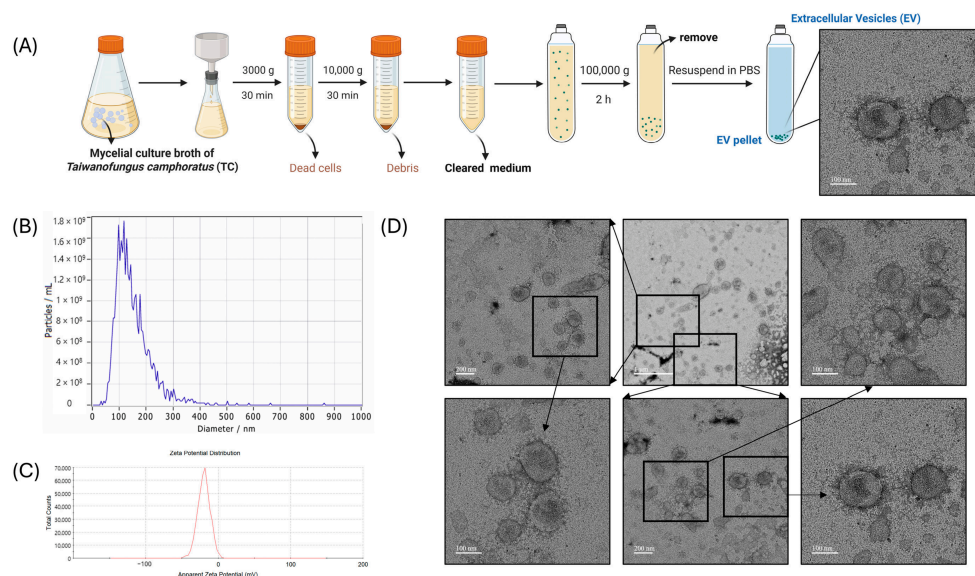


Figure 1. Isolation procedure and physicochemical characterization of *Taiwanofungus camphoratus*-derived extracellular vesicles (TCEVs). (A) Schematic illustration of the TCEV isolation procedure. After sequential centrifugation at $3000\times g$ and $10,000\times g$, the vesicle-containing pellet was collected by ultracentrifugation at $100,000\times g$ for 2 h and resuspended in PBS. (B) Particle-size distribution and concentration determined by nanoparticle tracking analysis. (C) Zeta potential and polydispersity index. (D) Representative TEM images of TCEVs. The rectangular boxes indicate the regions selected for magnification, and the arrows connect these regions to their corresponding enlarged TEM images. Scale bars are indicated in the corresponding panels. The scale-bar values are indicated in the corresponding TEM panels.

3.2. Effects of TCEVs on the Biosafety and Migratory Ability of HaCaT Cells

To evaluate cytocompatibility, HaCaT cells were treated with TCEVs for 24 h. Within the tested range of $0.625\text{--}10 \times 10^{10}$ particles/mL, cell viability remained above 90%, and no significant cytotoxicity was observed (Figure 2A). The scratch assay was conducted as an independent functional assessment of wound closure and migration-related activity rather than as a direct test of UVB protection. At 24 h, wound closure increased from 40.92% in the control group to 52.8% in the 10×10^{10} particles/mL TCEV group ($p < 0.001$; Figure 2B). The direct comparison between the highest-concentration TCEV group and the 25 μM resveratrol group was not statistically significant. Representative scratch images are shown in Figure 2C.

3.3. Effects of TCEVs on UVB-Induced Cellular Damage and Oxidative Stress

UVB irradiation at $150 \text{ mJ}/\text{cm}^2$ reduced cell viability to 66.46% and relative ATP content to 70.47% of the non-irradiated control. At 10×10^{10} particles/mL, TCEV pretreatment increased cell viability to approximately 80.0% and ATP content to 86.0%; both comparisons with the UVB group reached $p < 0.05$ (Figure 3A,B).

UVB irradiation increased relative intracellular ROS to 118.65%, whereas 10×10^{10} particles/mL TCEVs reduced ROS to 98.2% ($p < 0.01$ versus the UVB group; Figure 3C). UVB also decreased the JC-1 red/green fluorescence ratio to 92.37%, while the highest TCEV concentration increased it to 105.6% ($p < 0.001$ versus the UVB group; Figure 3D). In the representative JC-1 images, the UVB group showed weaker red aggregate fluorescence and relatively stronger green monomer fluorescence than the non-irradiated control. TCEV pretreatment increased red fluorescence and reduced the relative green signal, consistent with the quantified increase in the red/green ratio (Figure 3E).

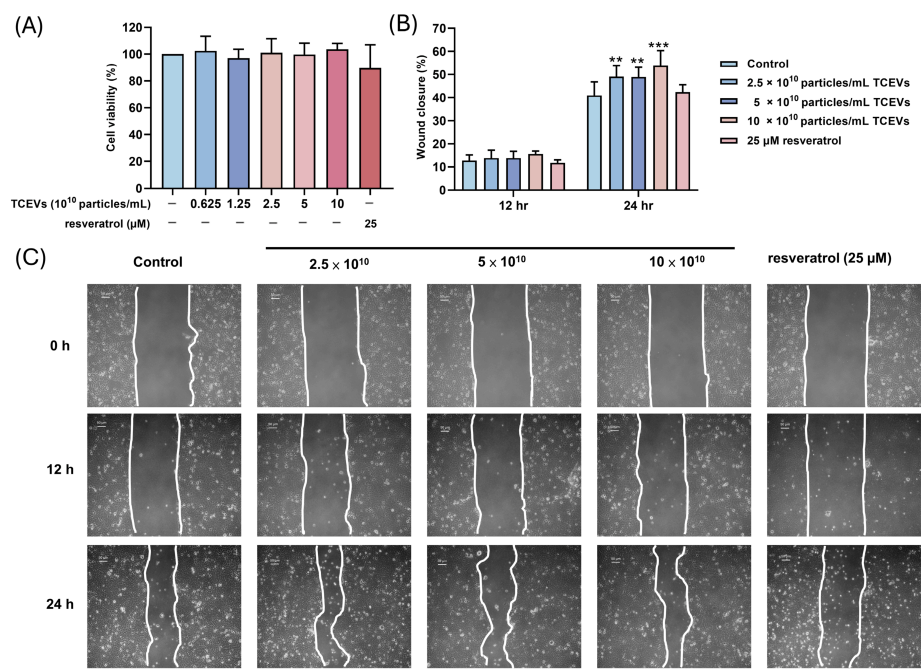


Figure 2. Effects of TCEVs on HaCaT cell viability and wound closure. **(A)** Cell viability after treatment with TCEVs (0.625, 1.25, 2.5, 5, and 10 $\times 10^{10}$ particles/mL) for 24 h; resveratrol (25 μ M) was used as the positive control. **(B)** Wound closure (%) at 12 and 24 h after treatment with TCEVs (2.5, 5, and 10 $\times 10^{10}$ particles/mL) or resveratrol (25 μ M). **(C)** Representative scratch-assay images at 0, 12, and 24 h. Data are presented as mean $\pm$ SD ($n = 3$). ** $p < 0.01$, *** $p < 0.001$ versus the control group. Scale bars = 50 μ m.

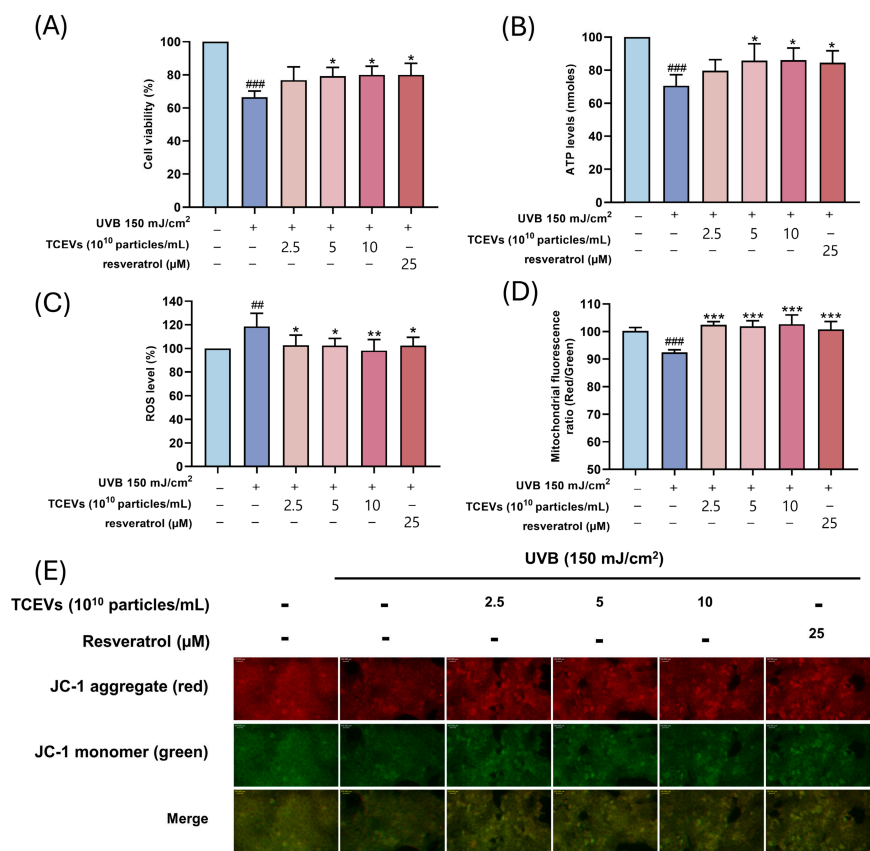


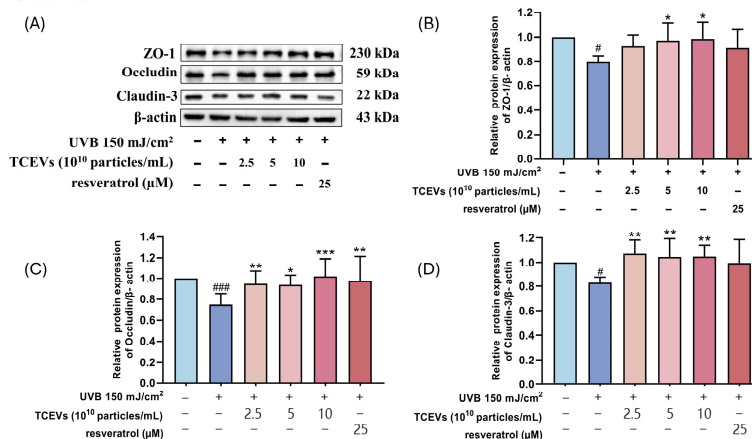
Figure 3. Effects of TCEVs on UVB-induced cellular damage, oxidative stress, and mitochondrial membrane potential in HaCaT cells. HaCaT cells were pretreated with TCEVs (2.5, 5, and 10 $\times 10^{10}$

particles/mL) for 6 h and then exposed to UVB (150 mJ/cm²), followed by incubation for 24 h. Resveratrol (25 µM) was used as an independent positive control. (A) Cell viability. (B) ATP levels. (C) ROS levels. (D) JC-1 red/green fluorescence ratio. (E) Representative JC-1 images: red fluorescence represents JC-1 aggregates associated with higher mitochondrial membrane potential, whereas green fluorescence represents JC-1 monomers associated with mitochondrial depolarization. The UVB group showed reduced red and relatively increased green fluorescence, while TCEV pretreatment shifted the signal toward increased red fluorescence. Data are presented as mean ± SD (n = 3). ## *p* < 0.01, ### *p* < 0.001 versus the non-irradiated control group; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 versus the UVB group. Scale bars = 100 µm.

3.4. Effects of TCEVs on Skin Barrier-Related Proteins

To evaluate barrier-related protein expression, ZO-1, occludin, and Claudin-3 were analyzed. UVB reduced the expression of these proteins, whereas selected TCEV groups showed significant improvement relative to the UVB group (Figure 4A–D). The magnitude of improvement was not uniformly linear across the tested concentrations.

Protein-TJ



Protein-Mitochondria

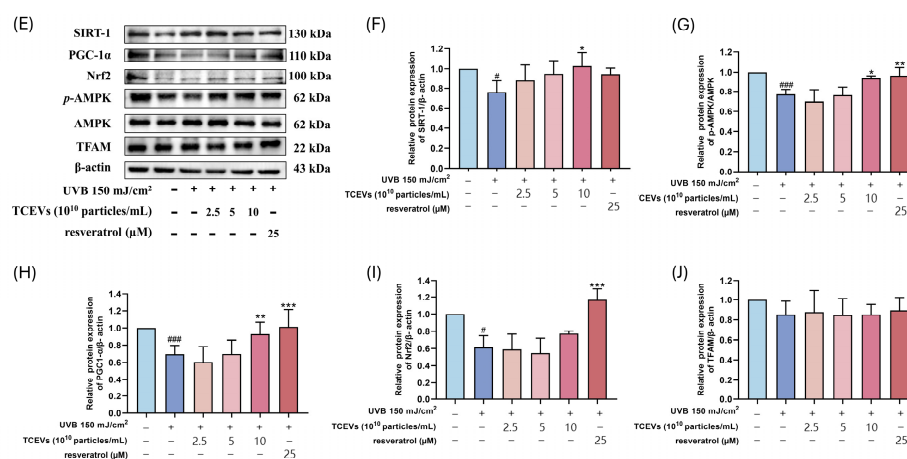


Figure 4. Effects of TCEVs on UVB-induced expression of epidermal barrier-related and mitochondria-related proteins in HaCaT cells. HaCaT cells were pretreated with TCEVs (2.5, 5, and 10 × 10¹⁰ particles/mL) for 6 h, exposed to UVB irradiation (150 mJ/cm²), and further incubated for 24 h. Resveratrol (25 µM) was used as the positive control. (A) Representative Western blot bands of epidermal barrier-related proteins, including ZO-1, occludin, Claudin-3, and β-actin. (B) Relative expression of ZO-1/β-actin. (C) Relative expression of occludin/β-actin. (D) Relative expression of Claudin-3/β-actin. (E) Representative Western blot bands of mitochondria-related proteins, including

SIRT1, PGC-1 α , Nrf2, p-AMPK, AMPK, TFAM, and β -actin. (F) Relative expression of SIRT1/ β -actin. (G) Relative expression of p-AMPK/AMPK. (H) Relative expression of PGC-1 α / β -actin. (I) Relative expression of Nrf2/ β -actin. (J) Relative expression of TFAM/ β -actin. Data are presented as mean $\pm$ SD ($n = 3$). # $p < 0.05$, ### $p < 0.001$ versus the non-irradiated control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the UVB group.

3.5. Effects of TCEVs on Mitochondria-Related Protein Expression

Western blot analysis was used to examine mitochondria-related regulatory proteins. TCEV treatment was associated with increased SIRT1 and p-AMPK expression relative to the UVB group; however, the magnitude of the response was not uniformly concentration-dependent (Figure 4E–G).

PGC-1 α expression was partially improved by TCEV treatment. In contrast, Nrf2 and TFAM expression did not differ significantly from the UVB-irradiated group under the present conditions, indicating that the measured mitochondrial regulatory proteins did not show a uniform recovery response (Figure 4H–J).

4. Discussion

UVB-induced photoaging involves oxidative stress, mitochondrial dysfunction, and disruption of epidermal barrier-related processes. In the present study, TCEVs were examined as a secreted vesicular fraction rather than as intact mycelia or crude extracts. This approach reduces direct exposure of HaCaT cells to intact fungal structures and bulk culture-medium components and permits treatment doses to be standardized by particle number. These features support the study of TCEVs as a distinct material, although the present study did not directly compare their safety or efficacy with whole fungi or crude extracts.

The pretreatment design was used to evaluate preventive and cytoprotective effects. UVB exposure increased ROS and reduced cell viability, ATP content, and mitochondrial membrane potential, whereas TCEV pretreatment reduced ROS accumulation and improved these functional outcomes. These results support protection against a subsequent UVB insult under the tested in vitro conditions rather than post-injury regeneration or treatment of established damage.

The improvements in JC-1 red/green ratio and ATP content were accompanied by increased SIRT1 and p-AMPK expression and partial recovery of PGC-1 α [30–36]. However, Nrf2 and TFAM did not differ significantly from the UVB group [37]. Thus, functional improvement was accompanied by selective rather than uniform changes in mitochondrial regulatory proteins. Because SIRT1 or AMPK inhibitors, gene knockdown, overexpression, or other functional-blocking approaches were not used, these changes should be interpreted as associative and do not demonstrate direct mediation of TCEV protection by the SIRT1/AMPK axis.

Across the tested concentrations of 2.5, 5, and 10×10^{10} particles/mL, the protective responses did not increase in a consistently linear or statistically distinct manner for every endpoint. This pattern is consistent with a plateau or non-linear dose–response in which a lower tested concentration may already produce much of the observable effects, and further increases in particle concentration provide limited additional improvement. The reason for this pattern cannot be determined from the present data.

The scratch assay and the UVB model provided complementary but distinct functional information. The scratch assay showed that TCEVs increased HaCaT wound closure, a migration-related process relevant to epidermal repair, whereas the UVB experiments evaluated the prevention of oxidative, mitochondrial, and barrier-related protein damage. Because the scratch assay was not performed after UVB irradiation, increased wound closure cannot be considered a demonstrated mechanism of UVB protection. Similarly, the

increases in ZO-1, occludin, and Claudin-3 support preservation of barrier-related protein expression, but overall barrier function was not directly established because TEWL or equivalent functional measurements were not performed.

Fungal EVs have been reported to contain proteins, lipids, carbohydrates, pigments, and RNA species [17–20]. *Taiwanofungus camphoratus* itself is known to produce triterpenoids, including antcin-related compounds, polysaccharides, and other secondary metabolites [21–25]. These molecules are therefore candidate TCEV cargos for future analysis, but their presence in the present TCEV preparation was not measured, and the observed cellular effects cannot be attributed to any specific compound. Future cargo profiling may combine LC–MS/MS proteomics, lipidomics, untargeted and targeted metabolomics, carbohydrate analysis, and RNA or small-RNA sequencing. NTA and TEM provide particle-size, concentration, and morphology information and do not establish a molecular cargo profile.

The present isolation data also have methodological limitations. Because the particle number was not measured before isolation, loss during individual centrifugation steps and overall recovery could not be calculated. Differential ultracentrifugation may co-isolate non-vesicular protein aggregates or other nanoscale material; therefore, NTA and TEM should be regarded as complementary physical characterization methods rather than proof of absolute purity [29]. Future purity assessment could incorporate density-gradient centrifugation or size-exclusion chromatography, EV-associated positive and negative markers, and particle-to-protein measurements.

The tested concentration range was selected after HaCaT cytocompatibility testing from 0.625 to 10×10^{10} particles/mL, in which viability remained above 90% at the highest concentration. The final TCEV stock concentration was approximately 2.33×10^{13} particles/mL; simple volume calculations indicate that final concentrations of 2.5, 5, and 10×10^{10} particles/mL would require approximately 0.11%, 0.21%, and 0.43% (v/v) of this stock, respectively. These values indicate only theoretical volumetric feasibility and do not establish stability, skin penetration, or biological effectiveness in a cosmetic formulation. The current PBS suspension stored at -80°C does not represent a finished cosmetic product. Further studies should assess formulation stability across temperature, pH, preservatives, surfactants, light exposure, and storage time; skin penetration; scale-up; batch consistency; microbial quality; and safety endpoints such as irritation, sensitization, genotoxicity, repeated exposure, and formulation compatibility. Applicable TFDA quality, safety, and product information requirements would also need to be addressed before cosmetic use.

Overall, the present findings are limited to an in vitro HaCaT model and a single post-treatment measurement time. The identity of active cargo, causal signaling sequence, long-term safety, and translational performance remain unresolved. Multi-omics cargo analysis, pharmacological or genetic pathway-blocking experiments, skin-equivalent models, and subsequent in vivo or clinical studies will be required to define the mechanism and application potential of TCEVs.

5. Conclusions

This study isolated and physically characterized a nanosized, membrane-bound TCEV preparation from *Taiwanofungus camphoratus* mycelial culture supernatant. In a UVB-induced HaCaT cell model, TCEV pretreatment reduced ROS accumulation, improved mitochondrial membrane potential and ATP production, and improved the expression of selected mitochondrial regulatory and epidermal barrier-related proteins. The changes in SIRT1, p-AMPK, and PGC-1 α were associative, while Nrf2 and TFAM did not differ significantly from the UVB group. These results support the potential of TCEVs to prevent

UVB-induced keratinocyte damage under the present in vitro conditions. Further studies are required to establish active cargo, causal signaling mechanisms, preparation purity, skin penetration, formulation stability, and long-term safety.

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