



Research Article

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Involvement of Cathepsin K and Cathepsin L2 in the Digestion of Melanosome Complexes in Epidermal Keratinocytes by Fennel Extract

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Abstract

In healthy skin, melanosome complexes are digested along with keratinization in the epidermal keratinocytes. However, in senile lentigo, large melanosome complexes accumulate in the basal and spinous epidermal layers. Melanosome complexes are degraded by lysosomes in human epidermal keratinocytes. We investigated how fennel extract affects melanosome degradation in melanosome-containing cultured epidermal keratinocytes. We also investigated the potential of fennel extract to increase intracellular lysosomal digestive enzyme activity using immunostaining and gene expression analysis. The fennel extract reduced tyrosinase-related protein (TRP-1), a mature melanosome membrane protein, and increased cathepsin K and cathepsin L2 expression. The intracellular cathepsin and TRP-1 co-localization was observed in melanosome-containing keratinocytes. In a 3D human epidermis model, the fennel extract increased cathepsin K expression, mainly in the basal and spinous epidermal layers, as well as in the entire stratum granulosum and stratum corneum. Cathepsin L2 expression increased mainly in the basal and the spinous epidermal layers. These results suggest that the fennel extract-induced digestive enzymes were involved in melanosome complex membrane protein degradation and keratinization-related melanin dispersion. Moreover, melanosomes could be degraded in epidermal keratinocytes by promoting cathepsin K and cathepsin L2 activities. Therefore, fennel might prove effective in making senile pigmentation fade.

Keywords: Cathepsin; Fennel extract; Lightening; Lysosome; Melanosome digestion; Pigmented spots

Introduction

Epidermal melanin is important in the regulation of skin color and determines the wide variety of skin colors associated with ethnic diversity [1]. In healthy skin, melanosome complexes in the epidermis are digested along with keratinization. However, in senile lentigo, they accumulate as large melanosome complexes in the basal and spinous layers of the epidermis. Thus, the size, number, and distribution of melanosome complexes are the main determinants of skin color. Melanin is synthesized in melanosomes, which are the organelles found in the pigment cells (melanocytes) in the basal layer of the epidermis. In the melanosomes, tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA), and DOPA to dopaquinone, by the action of the enzyme tyrosinase. After dopaquinone formation, the eumelanin pathway proceeds as follows: first, dopachrome is converted to 5,6-dihydroxy-1H-indole (DHI) by spontaneous decarboxylation or to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) catalyzed by dopachrome tautomerase

(DCT) [2]. Melanogenesis can culminate in eumelanin by oxidative polymerization reactions following the formation of DHI and DHICA [2]. Tyrosinase, a melanin synthase, is synthesized separately from melanosomes and transported by vesicles to immature melanosomes. Tyrosinase-related protein-1 (TRP-1) is present in mature melanosomes and has been used as a marker for melanosomes [3,4]. The melanin synthesized near the nucleus of melanocytes accumulates in melanosomes, and the mature melanosomes are transported to the vicinity of the cell membrane via long-range bidirectional microtubules and directly under the cell membrane via short-range unidirectional actin fibers. Subsequently, it combines with the cell membrane, is transferred to the adjacent keratinocytes, and accumulates on the nucleus of the keratinocytes to form a nuclear cap (melanin cap) [5,6]. We also reported in an earlier study that large melanosome complexes accumulate in the keratinocytes of the basal and spinous layers [6], resulting in senile pigmented lesions.

Melanosome complexes are known to be degraded by lysosomes in normal human epidermal keratinocytes [7]. It has been reported that aspartic proteases and cysteine proteases are involved in the promotion of tyrosinase degradation in acidic melanosomes [8]. Cysteine proteases are a type of acidic protease, and cathepsin L2 is considered to act as a cysteine protease in the degradation of tyrosinase in melanosomes. Studies on racial and skin color differences have reported that melanosomes of light-skinned keratinocytes are degraded faster than those of dark-skinned keratinocytes [9] and the activity of the enzyme cathepsin L2 is reported to be higher in light-skinned keratinocytes than in dark-skinned keratinocytes [10]. Because cathepsin K is also a cysteine protease, it is thought to have an effect of degrading tyrosinase in melanosomes. In Japan, medicated whitening cosmetics (“quasi-drugs”) that inhibit tyrosinase activity, such as kojic acid, arbutin [11], and rucinol, promote the degradation of tyrosinase, such as linoleic acid [12]. In addition, stable vitamin C derivatives, vitamin C ethyl with antioxidant properties [13], and anti-inflammatory properties, such as that in tranexamic acid [14], are being researched and developed [15]. However, agents such as rhododendrol and magnolignan are currently not used because they release hydroxyl radicals from tyrosinase, are highly cytotoxic to melanocytes, and may induce leukoderma [16]. The use of such substances, wherein hydroxyl radicals are generated by tyrosinase to regulate melanin production, poses a risk of causing vitiligo. Therefore, there is a growing need for developing skin-whitening ingredients with a mechanism that promotes the degradation of melanosome complexes accumulated in keratinocytes.

Fennel (scientific name: *Foeniculum vulgare* Mill.) is a perennial herb belonging to the Apiaceae (Umbelliferae) family. The young leaves and seeds (fennel seeds) have a sweet aroma and bitter taste, and effectively promote digestion and deodorizing. Since ancient times, fennel has been used as a spice and herb for food and medicinal purposes. The constituents of fennel seed oil are (E)-anethole, limonene, methyl chavicol, fenchone, α -pinene, and (Z)- β -ocimene [17,18]. Fennel seed oil reportedly exhibits anti-inflammatory properties [17]. In addition, fennel seed oil and seed extract in methanol or ethanol reportedly display antioxidant properties (DPPH scavenging activity, ferric reducing activity, and inhibition of peroxidation of linoleic acid) [19-21]. The composition of fennel seed methanolic extracts was characterized by its richness in quinic acid, 4-O-caffeoylquinic acid, p-coumaric acid, and 4-O-caffeoylquinic acid [20]. The water extract of fennel fruits also reportedly exhibits strong antioxidant activity (inhibition of peroxidation of linoleic acid) [21-23], and 4-O- β -D-glucosyl sinapyl alcohol, 4, 9-di-O- β -glucosyl alcohol, and 4- β -glucosyloxy benzoic acid were identified as its active components [23]. Based on this background, we investigated the degradation of melanosome complexes in cultured human keratinocytes by fennel extract, a natural component with reportedly strong antioxidant properties and analyzed the mechanism by focusing on lysosomes effects.

Materials and Methods

Materials

Dried fennel fruit (*Foeniculum vulgare* Miller fruits; Place of origin: China), sold as a crude drug, was purchased from Nakaya Hikojuro Pharmacy Co. Ltd. (Ishikawa, Japan). Anti-tyrosinase-related protein-1 (TRP-1) antibody, TMH-2 [24], was used. Anti-cathepsin K and anti-cathepsin L2 antibodies were purchased from BioVendor Laboratory Medicine, Inc. (Brno, Czech Republic) and Abcam plc. (Cambridge, United Kingdom). Alexa Fluor 488 goat anti-mouse IgG antibody, Alexa Fluor 488 goat anti-rabbit IgG antibody, Alexa Fluor 546 goat anti-rabbit IgG antibody, Alexa Fluor 546 goat anti-rat IgG antibody, Alexa Fluor 647 goat anti-rat IgG antibody, Lyso Tracker Green DND-26, Lyso Tracker RED DND-99, and Hoechst 33342 Nuclear Stain were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The other reagents used in the experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan). HaCaT cells and B16 melanoma cells were purchased from Cell Lines Service GmbH (Eppelheim, Germany). Human epidermal 3D models were purchased from Japan Tissue Engineering Co., Ltd (Aichi, Japan).

Fennel extract preparation

Thirty grams of fennel was soaked in 300 mL of 0%, 25%, 50%, 75%, and 99.5% ethanol solution for one week. The solvent was removed using a centrifugal concentrator (Savant SpeedVac Concentrators, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Bio Freeze Dryer (BFD-6F2, Nihon Freezer Co., Ltd., Tokyo, Japan); then, the yield was determined. The inhibition of L-DOPA autoxidation and the promotion of melanosome degradation in keratinocytes were measured as follows: First, L-DOPA autoxidation was measured by adding 90 μ L of a PBS solution (pH 7.4) of 10 mmol/L L-DOPA to the wells of a 96-well microplate. Then, 10 μ L of fennel extract was added with concentrations of 0 (control), 1.25, 2.5, and 5 mg/mL and incubated at 37°C for 2 days. The change in absorption at 550 nm was measured and used to quantify the L-DOPA autoxidation relative to the control. Second, for measuring the melanosome degradation-promoting effect, 20,000 cells/99 μ L of melanosome-containing and melanosome-free HaCaT cells were seeded in the wells of a 96-well microplate. Then, 1 μ L of fennel extract was added with concentrations of 0 (control), 1.25, 2.5, and 5 mg/mL and incubated at 37°C for 4 days. The absorption at 550 nm was measured and used to calculate the ratio of melanosome-containing to melanosome-free HaCaT cells. The absorption value for each fennel extract trial relative to the control was calculated; this value represents the extent of degradation in melanosome-containing keratinocytes. Both experiments were conducted with n = 3.

Effect of Fennel Extract on the Proliferation of HaCaT Cells and B16 Melanoma Cells

A fennel extract solution of 10 mg/mL was prepared by adding 1 mL of dimethyl sulfoxide (DMSO) to 10 mg of fennel extract

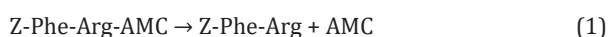
and diluted to 5 mg/mL with DMSO. Cells cultured in 10% FBS-DMEM (Fetal Bovine Serum-Dulbecco's Modified Eagle Medium) at 200,000 cells/mL, seeded in 96-well plates (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at 99 μ L/well, and cultured for 1 day. Then, 1 μ L of the control (DMSO) and fennel extract were added and cultured in 2% FBS-DMEM medium for 2 days. Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added at 10 μ L/well and cultured for 2 h, and the absorbance at 450 nm was measured with a microplate reader (Power Scan HT, BioTek, Winooski, VT, USA) and used as a measure of the cell count. In this method, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt]], which produces highly sensitive water-soluble formazan, was used. The number of living cells was determined by measuring the absorbance at 450 nm of the water-soluble formazan produced after reduction by intracellular dehydrogenase. There is a linear proportional relationship between the number of cells and the amount of formazan produced. The experiment was repeated three times (n=3).

Melanosomal Lysis and Absorbance Measurement

The medium in the 100 mm dish to which control, 15 μ g/mL and, 30 μ g/mL of fennel extract and 15 μ mol/L of chloroquine had been added was removed. Trypsin-EDTA (10 mL) was added and left in a 5% CO₂ incubator for 30 min, and the cells were collected in a 15 mL centrifuge tube and counted. The medium in the centrifuge tube was removed, the cell count was measured, and 1 mol/L NaOH was added according to the number of cells. In a 96-well plate, 100 μ L of each sample was added and the absorption spectra from 400 nm to 700 nm and the value at 550 nm were measured.

Effect of Fennel Extract on Cathepsin K and Cathepsin L2 in Keratinocytes

A solution of 4 mmol/L acetate buffer (pH 5.6), phosphate-buffered saline (PBS; pH7.4), 1 mmol/L cathepsin K substrate, 0.1 mmol/L cathepsin L substrate, and cathepsin B inhibitor solution was prepared. Cathepsin L2 specifically degrades the substrate Z-Phe-Arg-AMC and releases the fluorescent substrate 4-methylcumaryl-7-amide (AMC) [25,26]. The reaction equation is shown in Equation (1).



The fluorescence intensity of AMC was detected using a fluorescence microplate reader (Power Scan HT) at 37°C. As Z-Phe-Arg-AMC is also a substrate of cathepsin B, cathepsin L2 activity was specifically measured by mixing the reaction solution with [L-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline (CA-074), a specific inhibitor of cathepsin B [27]. The cells were prepared at 200,000 cells/mL, and seeded at 99 μ L/well in 96-well plates, and cultured for 1 day. After 3 days of culture, the medium on the 96-well plate was removed and discarded. The cell plates were washed twice with PBS, and frozen. After thawing, (1) 10 μ L of 1 mmol/L cathepsin K substrate and 90 μ L of PBS buffer were added to each well, or (2) 10 μ L of 0.1 mmol/L cathepsin

L2 substrate, 10 μ L of cathepsin B inhibitor solution, and 80 μ L of 4 mmol/L acetate buffer (pH 5.6) were added to each well. The fluorescence intensity (excitation, 360 nm, emission, 460 nm) was measured with a microplate reader, and the plate was placed in an incubator at 37°C for 4 h. The activities of cathepsin K and cathepsin L2 were measured over 4 h. The experiment was repeated three times (n=3).

Effect of Fennel Extract on mRNA Expressions of Cathepsin K and Cathepsin L2

HaCaT cells were seeded in 35 mm dishes at 0.999 mL/dish (30,000 cells/mL) and cultured for 1 day. Fennel extract was dissolved in DMSO to prepare solutions of 25 mg/mL and 50 mg/mL, and 1 μ L of extract was added to each dishes and cultured for 3 days (n=3). RNA was extracted from the cultured cells using an RNA extraction kit (RNeasy Mini Kit). A real-time PCR system was used to measure the relative mRNA expression levels of CTSK, CTSL2, and GAPDH. Primers were obtained from Qiagen N.V. (Venlo, The Netherlands).

Confocal Laser Scanning Microscopy Analysis of the Effects of Fennel Extract on Lysosomes and TRP-1 in Melanosome-Containing Keratinocytes

HaCaT cell suspensions (70,000 cells/mL) containing melanosomes were prepared, and 1 mL of the suspension was added to each chamber slide and cultured for 1 day. The fennel extract was dissolved in DMSO to prepare solutions of 6.25, 12.5, and 25 mg/mL, 1 μ L was added per chamber and cultured for 3 days. The chamber slides were washed with serum-free DMEM, and fluorescence reagent (Lyso Tracker Green DND-26 diluted 60,000-fold in 10% FBS-DMEM medium) was added (500 μ L per slide). After collecting the fluorescence reagent and washing with PBS, 500 μ L of 4% PFA was added for 10 min at 4°C to fix the cells. After fixation, the cells were washed with PBS and incubated with 100 μ L of 10% goat serum-PBS solution for 60 min. The cells were washed five times with PBS and then permeabilized with 0.1% Triton X-100 for 30 min. The cells were washed four times with PBS solution. The primary antibody (anti-TRP-1 antibody) was added and the cells were incubated for 2 h at room temperature. The cells were washed three times with PBS, and then 2000-fold diluted Alexa Fluor 546 goat anti-rat IgG antibody was added for 1 h at room temperature. The nuclei of the cells were stained and observed with the confocal laser scanning microscope.

Confocal Laser Scanning Microscopy Analysis of the Effects of Fennel Extract on Lysosomes and Cathepsin L2 in Melanosome-Containing Keratinocytes

Melanosome-incorporated cells were added to chamber slides at 500 μ L/well (100,000 cells/mL) and cultured for 2 days. Then, 5 μ L of solution was added so that the final concentrations of fennel extract and chloroquine were 15 μ g/mL, 30 μ g/mL, and 15 μ mol/L, and the cells were cultured for 2 days. As a control, 5 μ L of DMSO was added. The chamber slides were washed with serum-free DMEM, and the fluorescence reagent (LysoTracker Green DND-99 diluted 60,000 times in 10% FBS-DMEM medium) was prepared

and added to the slides (500 μ L per slide), and cultured for 60 min. After removing the fluorescence reagent, the slides were washed with PBS solution, and 500 μ L of 4% paraformaldehyde (PFA) in phosphate buffer was added for 10 min at 4°C to fix the cells. After fixation, the cells were washed with PBS and incubated with 100 μ L of 10% goat serum-PBS solution for 60 min. The cells were washed five times with PBS solution and permeabilized with 0.1% Triton X-100 for 5 min. The cells were washed four times with PBS solution. The primary antibody (anti-cathepsin L2 antibody) was then added, and the cells were incubated for 2 h at room temperature. The cells were washed with PBS solution three times, then Alexa Fluor 546 goat anti-rabbit IgG antibody (1:2000 dilution) was added for 1 h at room temperature. The stained cells were examined under a fluorescence microscope (FV3000, Olympus, Tokyo, Japan).

Cathepsin K, Cathepsin L2, and TRP-1 Staining in Normal Human Epidermal Keratinocytes (NHEKs)

Normal human epidermal keratinocytes (NHEKs, Kurabo Industries Ltd., Osaka, Japan) were cultured in a 75 cm² tissue culture flask in a CO₂ incubator with HuMedia-KG2 (Kurabo), a serum-free medium for tissue culture. After the culture medium was discarded, and melanosomes suspended in the medium were added, the NHEKs were cultured for 1 day to allow melanosomes incorporation. The medium used for the melanosome-incorporated NHEKs was discarded, and the cells were incubated with trypsin/EDTA solution for 10 min in a CO₂ incubator. The cells were then seeded into 35 mm dishes with 1 mL of trypsin/EDTA solution and incubated in the CO₂ incubator for 1 day. The cells were incubated in the CO₂ incubator for 2 days, and then the final concentrations of fennel extract were 15 μ g/mL and 30 μ g/mL. DMSO was added as a control. Staining for cathepsin L2, cathepsin K, and TRP-1 was performed as described in previous sections. Cathepsin K was stained used by anti-cathepsin K mouse monoclonal antibody (Santa Cruz Biotechnology Inc. Dallas, TX, USA). Alexa Fluor 647 goat anti-rat IgG antibody was used as the secondary antibody for TRP-1 staining.

Immunohistochemical Analysis of Fennel Extract on the Expression of Cathepsin K and Cathepsin L in the 3D models of Human Epidermis

The human epidermis models were cultured in a dedicated medium in the presence (12.5, 25, and 50 μ g/mL) and absence of fennel extract for 5 days, then embedded in a Tissue-Tek optimum cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The sections were incubated in 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4) for 10 min at 4°C, washed three times with PBS, then incubated with 10% goat serum in PBS for 1 h at room temperature in a humid chamber. After three washes with PBS, the sections were incubated overnight at 4°C with anti-cathepsin K and anti-cathepsin L2 antibodies diluted 500 \times in PBS. The sections were washed three times with PBS

supplemented with 0.05% Tween 20, then incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (1:2000 dilution) or Alexa Fluor 488 goat anti-rabbit IgG antibody (1:2000 dilution) at 37°C for 30 min in the humid chamber. Following one wash with PBS, the sections were mounted onto slides using Fluoromount (Japan Tanner Corporation, Osaka, Japan), covered with a cover glass, and observed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan). The fluorescence intensity and fluorescence area of three tissue sections were quantified using ImageJ (NIH, Bethesda, MD, USA). The fluorescent area/total area \times 100 was used as the area ratio, and the results of the area ratio \times fluorescence intensity calculation were compared.

Statistical Analysis

Measurements were analyzed for significant differences using the paired t-test function (two-sided) in Microsoft Excel. A P value of < 0.05 was considered statistically significant.

Results

Fennel extraction, L-DOPA autoxidation, and melanosome degradation

The results are summarized in Table 1. The highest extract yield was obtained using 99.5% ethanol and was in the form of an oil. The extract obtained from 75% ethanol was in the form of a plaque, whereas the extracts from 50%, 25%, and 0% ethanol were in the form of dry powders. The autoxidation of L-DOPA was significantly inhibited by the extracts from the 50% ethanol solution; however, it was promoted by the extracts from the 0% ethanol solution. The extracts from the 50% ethanol solution had the strongest effect on melanosome degradation in melanosome-containing keratinocytes. Subsequent experiments were carried out using fennel extracts from the 50% ethanol solution.

Effect of Fennel Extract on the Proliferation of HaCaT Cells and B16 Melanoma Cells

After 2 days of culture, the fennel extract had no statistically significant effect on the proliferation of B16 melanoma cells or HaCaT cells at any concentration added (25, 50, and 100 μ g/mL) and had no effect on cell proliferation at concentrations below 100 μ g/mL (Figure 1(a) & (b)).

Effect of Addition of Fennel Extract and Chloroquine on the Absorbance of Melanin in Cultured Melanosome-Containing Keratinocytes

The absorption spectra of the cells lysed in 1 mol/L NaOH and the values at 550 nm are shown in Figure 2. Compared with the control, the absorbance was lower in the presence of 15 μ g/mL and 30 μ g/mL of fennel extract, and higher in the presence of 15 μ M chloroquine. Compared with the control, the absorbance at 550 nm was lower in the presence of fennel extract and higher in the presence of chloroquine (Figure 2).

Table 1: Calculated yields of fennel extractions, inhibitory effect on the autoxidation of L-DOPA, and enhancement of melanosome degradation in melanosome-containing keratinocytes as a function of ethanol concentration.

Extraction condition	Concentration	Ethanol (%)				
		99.5	75	50	25	0
Yield (%)		48.5	17.1	12.4	14.4	21.4
Oxidation of L-DOPA (relative to the control at 550 nm as 100)	0.125 mg/mL	85.1	88	89.2*	89	89.8
	0.25 mg/mL	85.7	95.2	91.3*	95.1	85.7
	0.5 mg/mL	83.8	82.9	94.3	108.5	129.9***
Melanosome degradation in keratinocytes (relative to the control at 550 nm as 100)	12.5 µg/mL	83.4	85.5	76.7*	78.4	76
	25 µg/mL	79.5	76.7	71.7*	75.4	72.1*
	50 µg/mL	70.3*	80.6	62.5**	90.1	88.7

Source: The oxidation and degradation results are expressed as the ratio of the absorbance value at 550 nm of each solvent extract to that of the control, and averaged across three trials. The p-values are indicated as *p < 0.05 vs. control, **p < 0.01 vs. control, ***p < 0.001 vs. control.

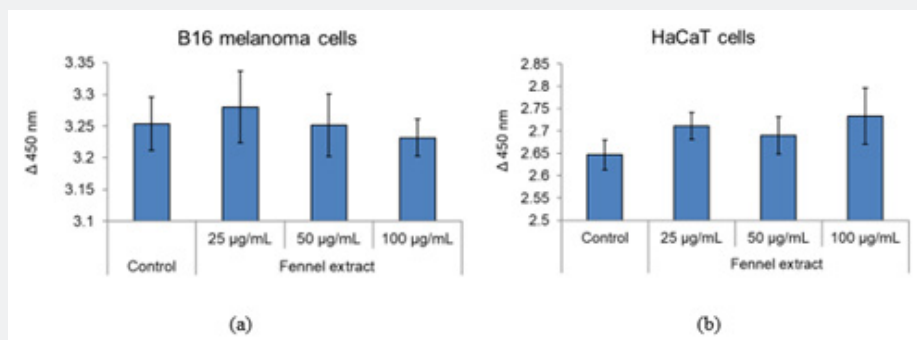


Figure 1: (a) Effect of fennel extract on the number of cultured B16 melanoma cells. (b) Effect of fennel extract on the number of cultured HaCaT cells. The results are expressed as the mean ± standard deviation of three experiments.

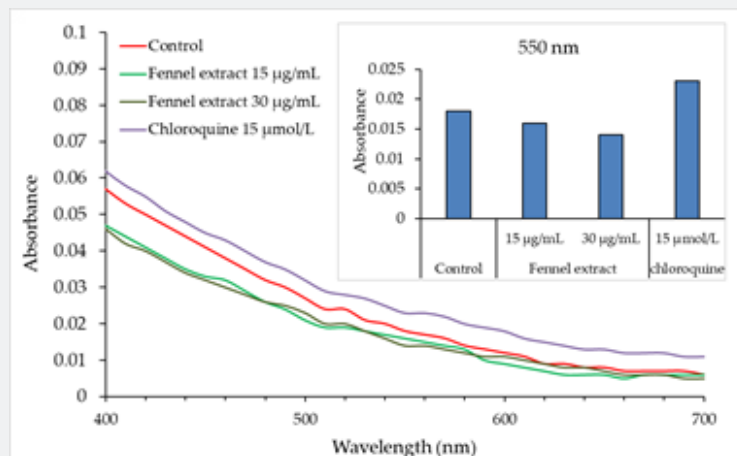


Figure 2: Effect of fennel extract addition and chloroquine addition on the color of melanin in cultured melanosome-containing keratinocytes.

Intracellular Localization of Cathepsin K and Cathepsin L

The intracellular localization of cathepsin K and cathepsin L was examined using immunofluorescent labeling. The localization of cathepsin K and lysosomes was consistent, but cathepsin L was found to exist in the cytoplasmic vesicles as well as lysosomes (Figure 3).

Effects of Fennel Extract on Cathepsin K and Cathepsin L2 in Keratinocytes

The effect of fennel extract on the activities of cathepsin K and cathepsin L2 in cultured keratinocytes was investigated at 25, 50, and 100 µg/mL, and it was found that the activities of these digestive enzymes were promoted higher than 25 µg/mL (Figure 4 (a) & (b)). We also examined the effect of fennel extract

on the mRNA expression of cathepsin K, cathepsin L2 in cultured keratinocytes. At 50 $\mu\text{g}/\text{mL}$, the fennel extract increased the expression of CTSK mRNA. The expression of CTSL2 mRNA was increased by 25 $\mu\text{g}/\text{mL}$ and 50 $\mu\text{g}/\text{mL}$ of fennel extract (Figure 4(c) & (d)).

Effects of Fennel Extract on Lysosomes and TRP-1 of Melanosome-Containing Keratinocytes Observed by Confocal Laser Scanning Microscopy

As it was found that fennel extract promoted the activity of intracellular cathepsin K, cathepsin L2, and the relative expression levels of human CTSK mRNA and CTSL2 mRNA, the effects of fennel extract on lysosomes and TRP-1 in melanosome-

containing keratinocytes were investigated using confocal laser scanning microscopy. The effects of fennel extract on lysosome and TRP-1 were visualized using immunostaining. We found that the fluorescence intensity of intracellular lysosomes (green) was stronger in the presence of fennel extract was added compared with the control solution (without fennel extract). Furthermore, the fluorescence intensity (red) of TRP-1 was weaker in cells in the presence of fennel extract (12.5, 25 $\mu\text{g}/\text{mL}$) compared with the control solution (without fennel extract). The fennel extract was found to promote the activity of lysosomes in the degradation of melanosomes, especially at concentrations of 12.5 $\mu\text{g}/\text{mL}$ and 25 $\mu\text{g}/\text{mL}$ (Figure 5).

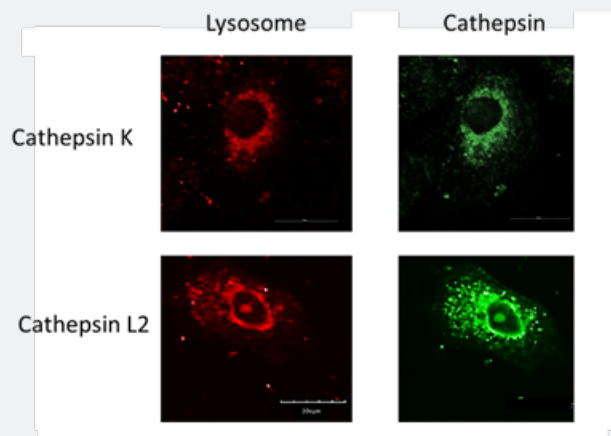


Figure 3: The intracellular localization of Cathepsin K and Cathepsin L observed by confocal laser scanning microscopy. The fluorescent intensity of intracellular lysosomes [LysoTracker Green DND-99 (red), a red fluorescent dye that stains acidic compartments in living cells]. Cathepsin K and Cathepsin L were labeled with antibodies of anti-cathepsin K and anti-cathepsin L2, and Alexa Fluor 488 secondary antibody. Scale bar = 20 μm .

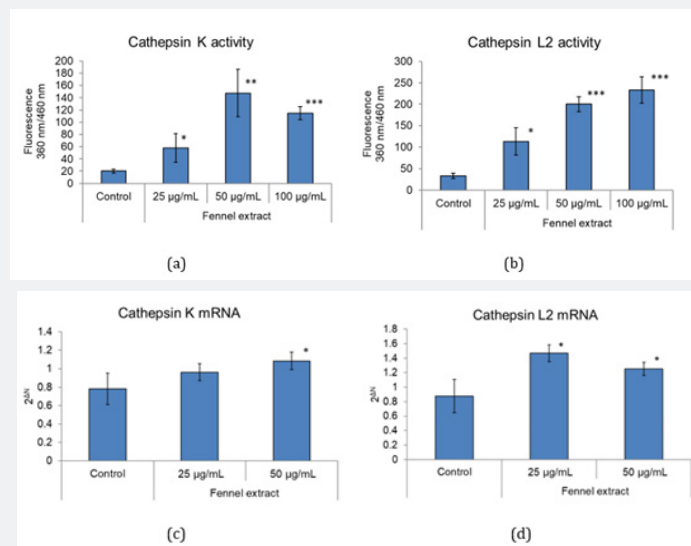


Figure 4: (a) Effect of fennel extract on cathepsin K in HaCaT cells. (b) Effect of fennel extract on cathepsin L2 in HaCaT cells. (c) Effects of fennel extract on relative mRNA expression level of cathepsin K (CTSK). (d) Effects of fennel extract on relative mRNA expression level of cathepsin L2 (CTSL2). The results are expressed as the mean \pm standard deviation of three experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control.

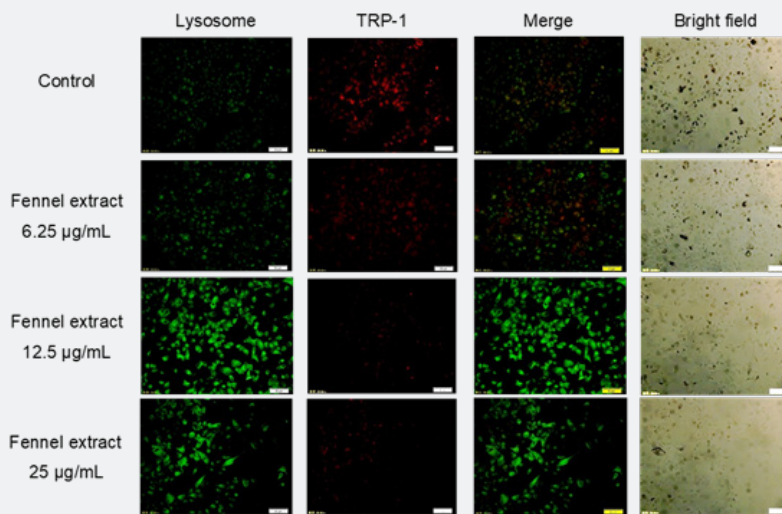


Figure 5: Effects of fennel extract on lysosomes and TRP-1 in melanosome-containing keratinocytes observed by confocal laser scanning microscopy. The fluorescence intensity of intracellular lysosomes [LysoTracker Green DND-26 (green), which is a green fluorescent dye that stains acidic compartments in live cells] was stronger in the presence of fennel extract compared with the control (without fennel extract). TRP-1, a membrane protein of melanosomes, was labeled with anti-TRP-1 antibody and Alexa 546 secondary antibody. Immunoreactive TRP-1 (red) was weakly expressed in the presence of fennel extract compared with the control (without fennel extract). The areas where lysosomal localization (green) and immunoreactive TRP-1 (red) coincide are shown in yellow in the merged color image. Scale bar = 50 µm.

Effects of Fennel Extract and Chloroquine on Cathepsin L2 in Melanosome-Containing Keratinocytes

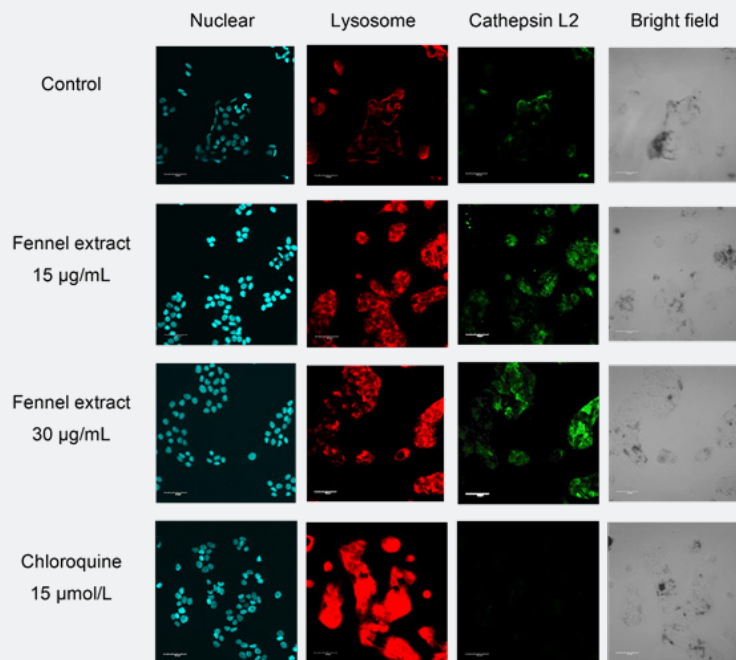


Figure 6: The effects of fennel extract and chloroquine on lysosomes in melanosome-containing keratinocytes were observed by confocal laser scanning microscopy. The lysosomes [LysoTracker DND-99 (red), a red fluorescent dye that stains acidic compartments in living cells] were more strongly stained in the presence of fennel extract (15 µg/mL) compared with the control (without fennel extract). With the addition of chloroquine, the fluorescence intensity of the lysosomes was stronger than in the presence of the fennel extract, Nuclei (blue), lysosomes (red). Scale bar = 50 µm.

We observed that 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ of fennel extract resulted in stronger fluorescence intensity of lysosomes and cathepsin L2 compared with the control (Figure 6). The addition of 15 $\mu\text{mol/L}$ of chloroquine resulted in significantly stronger fluorescence intensity of lysosomes than the addition of fennel extract (Figure 6). However, chloroquine markedly reduced the fluorescence intensity of cathepsin L2 (Figure 6). TRP-1 and cathepsin K in melanosome-incorporated NHEKs were stained with anti-TRP-1 and anti-cathepsin K antibodies after culture in medium containing fennel extract for 2 days. The results of observation by confocal scanning laser microscopy are shown in Figure 7. The fluorescence intensity of TRP-1 was lower

after treatment with 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ of fennel extract compared with the control. The fluorescence intensity of cathepsin K was increased after treatment with 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ of fennel extract compared with the control. The merged images of TRP-1 and cathepsin K showed that there was stronger staining of cathepsin K in the vicinity of the TRP-1 staining after treatment with 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ of fennel extract compared with the control. In the bright-field images, there were fewer melanosomes compared with the control. The co-localization of TRP-1 (blue) and cathepsin K (green) is shown in yellow in the merged, enlarged color image. The localization of TRP-1 was found to be consistent with that of cathepsin K in many places.

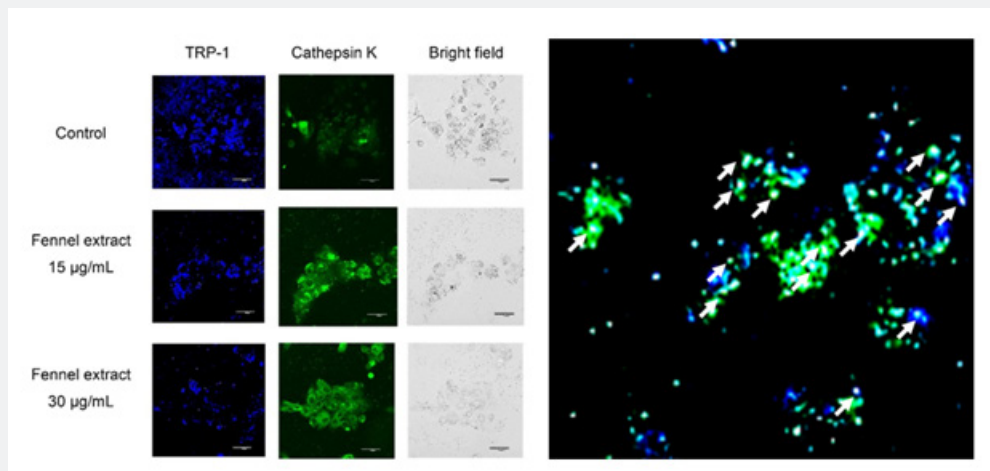


Figure 7: Confocal laser scanning microscopy images of TRP-1 and cathepsin K localization in melanosome-incorporated NHEKs cultured for 2 days in medium containing fennel extract. TRP-1 (blue), cathepsin K (green). Scale bar = 50 μm . The co-localization of TRP-1 (blue) and cathepsin K (green) is shown in yellow in the merged, enlarged color image. The white arrows indicate sites of co-localization.

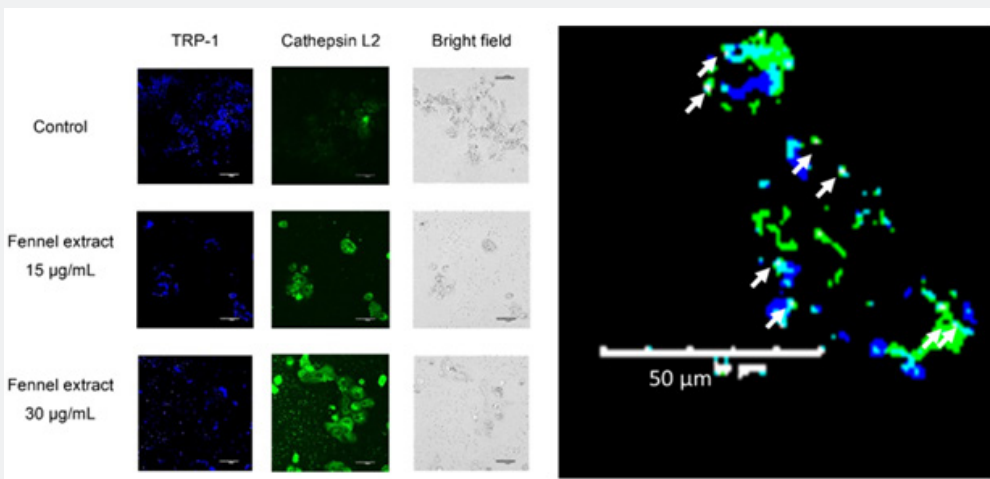


Figure 8: Confocal laser scanning microscopy images of TRP-1 and cathepsin L2 localization in melanosome-incorporated NHEKs cultured for 2 days in medium containing fennel extract. TRP-1 (blue), cathepsin L2 (green). Scale bar = 50 μm . The co-localization of TRP-1 (blue) and cathepsin L2 (green) is shown in yellow in the merged, enlarged color image. The white arrows indicate sites of co-localization.

TRP-1 and cathepsin L2 of melanosome-incorporated NHEKs were stained with anti-TRP-1 and anti-cathepsin L2 antibodies, respectively, and observed by confocal scanning laser microscopy when they were cultured in medium containing fennel extract for 2 days. The results are shown in Figure 8. In the presence of fennel extract 15 µg/mL and 30 µg/mL, the fluorescence intensity of TRP-1 was lower than that of the control. The fluorescence intensity of cathepsin L2 was higher in the presence of 15 µg/mL and 30 µg/mL of fennel extract compared with the control. The merged images

of TRP-1 and cathepsin L2 showed that the staining intensity of cathepsin L2 near the TRP-1 staining was stronger in the presence of 15 µg/mL and 30 µg/mL of fennel extract compared with the control. The bright-field images showed that there were fewer melanosomes in the presence of 15 µg/mL and 30 µg/mL fennel extract compared with the control. The co-localization of TRP-1 (blue) and cathepsin L2 (green) is shown in yellow in the merged, enlarged color image. There were also a few cases where the localization of TRP-1 coincided with that of cathepsin L2.

Effect of Fennel Extract on Cathepsin K and Cathepsin L2 Protein Levels in Human Epidermis 3D Models

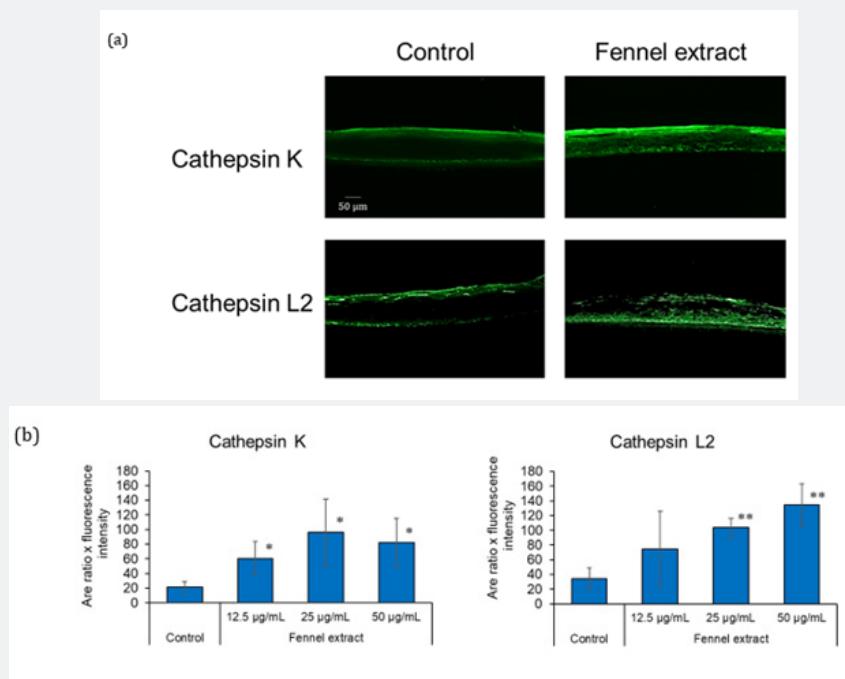


Figure 9: Effect of fennel extract on cathepsin K and cathepsin L2 protein levels in the models of the human epidermis. (a) The 3D models of the human epidermis were cultured with 50 µg/mL fennel extract for 5 days. Cathepsin K (green) and cathepsin L2 (green) were localized in the cellular cytoplasm in the basal and spinous layers. Scale bar = 50 µm. (b) The area ratio × fluorescence intensity of immunostained cathepsin K and cathepsin L2 in the presence and absence of the fennel extract (12.5, 25, and 50 µg/mL). The results are expressed as the mean ± standard deviation of three experiments. * p < 0.05 vs. control, ** p < 0.01 vs. control.

The effects of fennel extract on cathepsin K and cathepsin L2 protein levels in human epidermis 3D models were investigated through an immunohistochemical analysis. In the absence of fennel extract (control), cathepsin K and cathepsin L2 were localized predominantly in the basal and stratum corneum layers of the epidermis (Figure 9(a)). In the 3D human epidermis models cultured in the presence of fennel extract, cathepsin K was localized in the cellular cytoplasm in the basal and spinous layers and throughout the entire stratum granulosum and stratum corneum layers of the epidermis with higher protein level than in the control (Figure 9(a)). Cathepsin L2 was localized in the cellular cytoplasm in the basal and the spinous layers of the epidermis in the presence of fennel extract, and the protein level was higher than that in the control (Figure 9 (a)). The imaging analysis

revealed that immunoreactive cathepsin K was significantly increased in the presence of 12.5, 25, and 50 µg/mL fennel extract compared to the control (Figure 9 (b)). Immunoreactive cathepsin L2 was also significantly increased in the presence of 25 and 50 µg/mL fennel extract compared to the control (Figure 9(b)).

Discussion

The addition of fennel extract decreased the amount of melanin in cultured melanosome-containing keratinocytes compared with the control conditions (the absence of fennel extract). The protein level of TRP-1 present in the mature melanosomes was also reduced, suggesting that fennel extract promoted the degradation of the melanosome complex in keratinocytes. In the melanosome-containing keratinocytes, the lysosomal staining

intensity was enhanced by the addition of fennel extract. In addition, the amount of cathepsin L2 protein was increased and the intensity of fluorescent antibody staining was also enhanced. Therefore, it is thought that the extract activates lysosomes and cathepsin L2. It has been reported that cathepsin L2 is present close to melanosomes that have transferred to keratinocytes and is involved in the degradation of the melanosome complex. In melanosome-containing keratinocytes, the addition of 15 $\mu\text{mol/L}$ chloroquine resulted in stronger fluorescence intensity and a wider staining area of lysosomes compared with the control and the addition of fennel extract. However, the amount of melanin in the keratinocytes with melanosomes were incorporated was higher in the presence of chloroquine than in the control. Chloroquine is a lysosomotropic drug that raises intralysosomal pH [28] and impairs autophagic protein degradation [29]. Similarly, bafilomycin A1 has the effect of neutralizing the pH of acidic organelles, such as lysosomes, and it has been confirmed that bafilomycin A1 increases the amount of melanin [8]. The amount of melanin in melanosome-containing keratinocytes was higher than in the control in the presence of chloroquine, which may be due to the fact that chloroquine inhibited the degradation of melanosomes incorporated into keratinocytes or acidified lysosomes. Therefore, we hypothesized that melanosomes were degraded by the fusion of melanosomes and lysosomes, and further by the increased expressions of cathepsin K in lysosomes and cathepsin L2 in lysosomes and cytoplasm.

In melanosome-containing keratinocytes, as the staining intensity of the lysosomes was increased by the presence of the fennel extract. Studies on racial and skin color differences have reported that melanosomes from light-skinned keratinocytes are degraded faster than those from darker skin [9] and that the activity of the lysosomal enzyme cathepsin L2 is higher in light-skinned keratinocytes than in dark-skinned keratinocytes [10]. The effect of fennel extract on the activity of the intracellular digestive enzyme cathepsin K and cathepsin L2 was investigated, and it was found that fennel extract promoted the activities of cathepsin K and cathepsin L2. Therefore, the fennel extract may be effective in promoting the degradation of melanosomes in epidermal keratinocytes [8]. We also observed that the fennel extract increased the expressions of cathepsin K and cathepsin L mRNAs, which are the digestive enzymes of lysosomes. The inactive cathepsin precursor is synthesized in the endoplasmic reticulum and then transported to the Golgi apparatus, where it is further glycosylated and phosphorylated to form mannose-6-phosphate protein. Finally, the modified protein is recognized by the mannose-6-phosphate receptor in the lysosome, where the proenzyme is hydrolyzed at low pH and the prodomain is removed to produce active, mature cathepsin [30]. Therefore, the degree of increase in mRNA of cathepsin K and cathepsin L2 by fennel extract may have been lower than the degree of increase in their activity.

Furthermore, we observed the effect of fennel extract on the degradation of melanosomes in epidermal keratinocytes by confocal laser scanning microscopy, and confirmed that fennel extract reduced tyrosinase-related protein-1 (TRP-1), a melanosomal membrane protein. The localization of TRP-1 was found to be consistent with that of cathepsin K in many places. There were also a few cases where the localization of TRP-1 coincided with that of cathepsin L2. From these results, it was confirmed that fennel extract promoted the degradation of melanosomes in epidermal keratinocytes. In addition, cathepsin K and cathepsin L were thought to be involved in the degradation of melanosomes to some extent. Melanosomes phagocytosed by keratinocytes are present in keratinocytes as melanosome complexes. However, the membrane proteins are degraded through keratinization into complexes smaller than 400 nm and dispersed to become invisible. Nevertheless, in senile pigmented spots, multiple melanosome complexes remain in the basal and spinous layers [6]. In this study, applying a human epidermal 3D model, fennel extract increased the expression of cathepsin K mainly in the stratum granulosum and stratum corneum as well as in the basal layer and increased cathepsin L2 expression mainly in the spinous and basal layers. Therefore, these digestive enzymes might be involved in membrane protein degradation in the melanosome complexes and melanin dispersion by keratinization.

Based on the results herein, we believe that fennel extract can degrade melanosomes in epidermal keratinocytes by promoting the digestive enzymes, cathepsin K and cathepsin L2, and this is expected to be effective in helping senile pigmented spots fade.

Conclusion

Melanin amount and TRP-1 expression in mature melanosomes in melanosome-containing keratinocytes were decreased by the addition of fennel extract. Furthermore, cathepsin K and cathepsin L2 immunostaining intensity, activity, and mRNA expression increased. These results suggest that the fennel extract promoted melanosome degradation through the activation of the lysosomal digestive enzymes, cathepsin K and cathepsin L2. The intracellular co-localization of these cathepsins and TRP-1 was observed in melanosome-containing keratinocytes. In the 3D models of the human epidermis, cathepsin K expression was increased by the fennel extract, primarily in the stratum granulosum and stratum corneum but also in the basal layer, and cathepsin L2 expression was increased primarily in the spinous and basal layers. These results indicate that fennel extract can be effective in making senile pigmentation fade because it promotes the degradation of melanosomes from the basal layer to the stratum corneum of the epidermis by inducing cathepsin K and cathepsin L2 expression.

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