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Cosmetic potential of boiled water of Hijiki (*Sargassum fusiforme*) grown in the ocean in Okinawa, Japan

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Research

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Abstract

Okinawa has most beautiful ocean that attracts tourists and it was recorded over 10 million foreign tourists visited Okinawa in 2018. The ocean surrounding Okinawa has various species of marine algae that could be raw material for cosmetic products because it has high concentration of phenolic compounds contributing antioxidant activity. There is an urgent need to create Okinawa's unique brands for cosmetic products using marine algae grown in the ocean of Okinawa. Some of marine algae including popular seaweed food, Mozuku (*Cladosiphon okamuranus* Tokida) and Hijiki (*Sargassum fusiforme*), had high oxygen radical absorbance capacity and tyrosinase inhibition. In the food processing of Hijiki, there is a boiling step needed. And this Hijiki liquor is wasted 15 tons per year. To take advantage of this residue, development of cosmetic products derived from Hijiki liquor should be required. Hijiki liquor had the effect of decreasing melanin pigmentation and no cytotoxicity in a three-dimensional human skin model. Therefore, there is a high possibility that Hijiki liquor is a great material for cosmetic products.

Introduction

Okinawa is the southernmost prefecture of Japan and one of most popular beach resorts in Japan. In fact, over 10 million foreign tourists visited Okinawa in 2018, which is the highest number recording in any year. Okinawa prefecture consists of 160 large and small islands extending southwest from Kyushu to Taiwan. Okinawa has an independent and unique environment surrounded by the ocean. Furthermore, Okinawa has the coral reefs and crystal-clear blue seas, which let many kinds of marine algae grow there. Marine algae could be raw material for cosmetic or healthy food products. One of reasons is that the relatively high concentration of phenolic compounds in marine algae species contributes to their antioxidant properties, which can be of benefit in reducing oxidative reactions deleterious to health. (Freile-Pelegrín, 2013). There is an urgent need to create Okinawa's unique brands that attract tourists, using marine resources obtained on coast of Okinawa because there are unique species and some species have unique characteristics. Mozuku (*Cladosiphon okamuranus* Tokida) is a unique Okinawan seaweed food and it contains fucoxanthin, a kind of carotenoid, known for its many kinds of health benefits (Iwai et al, 2018; Maeda et al, 2018; Rokkaku et al, 2013). In addition, Hijiki (*Sargassum fusiforme*) grown in Okinawa is popular food as "Shimahijiki" that means Island Hijiki because it has unique characteristics having thicker stalk and being a bit more crunchy than those grown outside Okinawa in Japan. And Hijiki has some kinds of phenolic compounds such as phloroglucinol (Samee et al, 2009). In the food processing of Hijiki, there is a boiling step needed. In this step, all of cooking water (liquor) after boiling is currently wasted, which is 15 tons per year. Therefore, development of cosmetic product derived from Hijiki liquor is required to take advantage of this residue because it is expected to contain anti-aging or whitening components, such as polyphenols. In this study, we analyzed oxygen radical absorbance capacity (ORAC) and tyrosinase inhibition of some kinds of seaweeds obtained in Okinawa ocean and melanin decrease effect of Hijiki liquor on a three-dimensional human skin model.

Materials And Methods

Collecting seaweed sample

Seaweed samples were collected along the coast in each area (Table 1, Fig. 1). Each samples were several hundreds of gram (at least more than 200 g). Hijiki was obtained in Yonabaru town. And it was boiled by water in the food process at the factory in Yonabatu town (Fig. 2). The resulting boiled water (Hijiki liquor) was used for sample of the human skin model.

Powdering seaweed sample

Collected seaweed samples were freeze dried after being washed with water and was crushed for extraction using a grinder (MF10 basic, IKA, Staufen, Germany). Powdered seaweed samples were mixed enough until consistent.

Extraction for ORAC assay

Extraction process was achieved according to modified previous described (Mikami *et al.*, 2009)). A freeze-dried seaweed sample (100 mg) was added to a 10 mL screw-cap tube with 8 mL of hexane/dichloromethane (1:1, v/v) and shaken overnight by an orbital shaker, centrifuged at 2,000*g* for 10 min, and the supernatant was then collected. Residues were mixed twice with 2 mL of hexane/dichloromethane and were vortexed for 30 seconds, and the supernatant was collected by centrifugation at 2,000*g* for 10 min. These hexane/ dichloromethane fractions were dried by nitrogen gas and dissolved in 0.4 mL of acetone. This solution was then used for the lipophilic ORAC (L-ORAC) assay after having been appropriately diluted with acetone solvent. The residual was dried by using nitrogen gas. 4 mL of methanol/water/acetic acid (70:29.5:0.5, v/v/v) was then added, and the solution was shaken overnight by an orbital shaker. After being centrifuged at 2,000*g* for 10 min, the supernatant was used for the hydrophilic ORAC (H-ORAC) assay.

ORAC assay

ORAC value was evaluated as previously described (Mikami *et al.*, 2009). In summary, 110.7 nmol/L fluorescein sodium salt (SIGMA-ALDRICH, USA) solution and 31.7 mmol/L AAPH 2,2-azobis (2-amidinopropane) solution were prepared in 75 mmol/L phosphate buffer solution (pH 7.4). After dilution with assay buffer, sample solution was used for the ORAC assay. Diluted sample solution and fluorescein solution were applied to the microplate. Fluorescence (excitation, 485 nm and emission, 528 nm) was recorded for the first time after a 10 min incubation at 37°C in a Multi-Detection Microplate Reader SH-9000 (CORONA, Ibaraki, Japan) equipped with a temperature-controlled incubation chamber. The microplate was taken out of the SH-9000, and AAPH solution was added to the microplate, after which the plate was placed in the SH-9000 again. Fluorescence (excitation, 485 nm and emission, 528 nm) was then recorded every 2 min for 1.5 h using the SF6 software. Three measurements of duplicate data were expressed as average µmol of Trolox equivalents (TE) per 100 g of sample (µmol TE/g).

Tyrosinase inhibition

Inhibitory activities of seaweed samples on mushroom tyrosinase activity were evaluated on the basis of the method (Likhitwitayawuid et al. 2001) with slight modification. To a 96-well plate were added 20 μL of each sample solution (dissolved in 5% DMSO), 40 μL of mushroom tyrosinase (40 U/mL, Sigma-Aldrich Corp.), and 100 μL of 67 mM phosphate buffer (pH 6.8). After preincubation at 23°C for 3 min, 50 μL of 2.5 mM L-DOPA was added in the 96-well plate, and the optical density at 490 nm of each well was measured using a microplate reader (model 550, Bio-Rad Laboratories, Inc., Richmond, CA). After incubation at 23°C for 10 min, the increase in the optical density at 490 nm was measured. The percentage inhibition of tyrosinase activity was calculated by the following equation: % inhibition (%) = [1 - (A - B)/C] × 100, where A is the optical density at 490 nm with test sample and enzyme, B is the optical density at 490 nm with the test sample and without enzyme, and C is the optical density at 490 nm with enzyme without test sample. Three measurements of duplicate data were expressed as average %.

Fractionation

Hijiki liquor was purified using open glass column tube (80 mmI.D. × 300 mm) filled with DIAION HP20 (HP20) (Mitsubishi Chemical Co. Ltd., Tokyo, Japan), flowed with special grade methanol (MeOH) (NACALAI TESQUE, INC., Kyoto, Japan). HP20 is styrene-divinylbenzene synthetic adsorbent and is used for polyphenol isolation (Monsanto et al., 2015).

Determination of IC_{50} for tyrosinase inhibition

The extent of inhibition upon Hijiki liquor is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC_{50}). The concentration of inhibitor needed to inhibit 50% of the tyrosinase activity was extrapolated from % activity vs. [the sample] curves.

Culture for the human skin model.

The three-dimensional (3D) human skin model was placed on a 6-well plate with 0.9 mL of medium and incubated at 37°C for 1 h in a humidified atmosphere, which contained 5% CO_2 in air. After incubation, the skin model was placed on another 6-well plate that contained 5 mL of fresh medium. An aliquot (0.1 mL) of samples of 5, 10, and 20 mg/mL of MeOH fractionation dissolved in 12.5% glycerol and that of positive control (1 mg/mL of arbutin) were applied on the surface of the tissue on model cup (n = 4 except positive control, n = 2). The tissue was cultured for 10 days and refed with 0.1 mL of the sample solution and fresh LLMM every 2 days. At the end of culture, the melanin production and the viability of the tissue cells were measured.

Melanin quantitation for the human skin model.

The melanin production of the tissue cells of the three-dimensional (3D) cultured human skin model was measured using a minor modification to the method (Bessou-Touya et al., 1998) At the end of culture, the skin model was placed on a 24-well plate, and the tissue surface was rinsed three times with

DulbeccoPBS. The tissue was treated with 0.45 mL of 10 mM Tris-HCl buffer (pH6.8, which contained 1% SDS and 0.05 mM EDTA) and incubated at room temperature for 3 h in an airtight container with 20 μL of 5 mg/mL proteinase K. Following the incubation, the tissue was transferred from the model cup to a 1.5 mL tube and again incubated at 45°C overnight. To remove coloring matter, the tube was centrifuged at 20,000*g* for 15 min, and the supernatant was discarded. The precipitated tissue was washed with 0.45 mL of 10 mM Tris-HCl (pH 6.8, which contained 0.05 mM EDTA), and the supernatant was removed after centrifugation. The washed tissue was reacted with 20 μL of 5 mg/mL proteinase K and 0.45 mL of 10 mM Tris-HCl (pH 6.8, which consisted of 1% SDS and 0.05 mM EDTA). The obtained lysate was mixed with 0.5 M sodium carbonate (50 μL) and 30% hydrogen peroxide (10 μL), incubated at

80°C for 30 min, and then allowed to cool. The lipid from the tissue was removed by the addition of 100 μL of a chloroform/methanol (2:1) mixture followed by centrifugation at 10,000*g* for 10 min. The optical density of the aqueous phase was measured at 405 nm on a 96-well plate with a microplate reader (model 680, Bio-Rad).

Cell viability of the human skin model.

The viability of the tissue cells of the 3D human skin models was determined with the MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] dye reduction assay. At the end of culture, the model cup was rinsed three times with Dulbecco-PBS. The skin model was placed on a 24-well plate, 300 μL of MTT solution (MTT-100 kit, Kurabo) was added, and the tissue was incubated at 37°C for 3 h in a humidified atmosphere, which contained 5% CO2. After incubation, the tissue was washed with

Dulbecco-PBS. The skin model was placed on another 24-well plate, 2 mL of MTT extraction solution (provided with the kit) was added to each well, and the plate was shaken at room temperature for 2 h. The optical density of 200 μL of the extract was measured at 570 nm on a 96-well plate with a microplate reader (model 680).

Results And Discussion

ORAC assay and Tyrosinase inhibition

Padina minor that is brown algae, had the highest H-ORAC (299.6 μmol TE/g) and total ORAC (324.6 μmol TE/g) values among seaweeds we obtained (Table 1). And *Halimeda incrassata* is green algae, had the second highest H-ORAC (190.9 μmol TE/g) and total ORAC (201.4 μmol TE/g) values. Among red algae, *Aeanthophora spicifera* had the highest H-ORAC (115.9 μmol TE/g) and total ORAC (119.7 μmol TE/g) values. In terms of L-ORAC, *Caulerpa serrulate*, green algae, had the highest value (87.5 μmol TE/g), followed by *Turbinaria ornata*, brown algae (80.7 μmol TE/g) and *Dictyopteris latiuscula*, brown algae (55.9 μmol TE/g). Interestingly, they had higher L-ORAC values than H-ORAC values. L-ORAC stems from α-tocopherol, (+)-γ-tocopherol, (+)-δ-tocopherol, α-tocopherol acetate, tocotrienols (vitamin E group), 2,6-di-tert-butyl-4-methylphenol, and γ-oryzanol (Huang et al., 2002) while H-ORAC values well correlated with polyphenol content (Milan et al., 2010). As a result, these three species must be utilized as vitamin E

and other source because they had high L-ORAC values, compared to other seaweeds in this study. Besides, Hijiki (*Sargassum fusiforme*) and Mozuku (*Cladosiphon okamuranus* Tokida) which are popular as local food in Okinawa, had high H-ORAC values (124.4 μmol TE/g and 103.1 μmol TE/g). Also, it's interesting that Hijiki (*Sargassum fusiforme*) and Mozuku (*Cladosiphon okamuranus* Tokida) had the inhibitory effects of the tyrosinase activity (61.0% and 78.2%). They are expected to be nutritional functional food and also hoped to be used for cosmetic products. In terms of tyrosinase inhibition, *Dictyopteris latiuscula* had 99.4% tyrosinase inhibition, *Hormophysa cuneiformis* at 96.5%, and *Padina minor* at 89.2%, which are all brown algae. Neverthless, they haven't been eaten in the history. Accordingly, there is a need to do further study about cytotoxicity tests and some others in a case that they are used for cosmetic products. Although *Halimeda incrassate*, *Caulerpa cupressoides*, and *Caulerpa serrulate* (green algae) had high total ORAC values, they didn't have tyrosinase inhibition. The inhibition of mushroom tyrosinase is caused by the hydrophobic p-alkyl group (Xiao-Hong et al., 2006) and the unsaturated fatty acids, such as (2E, 4E)-hexa-2,4-dienoic acid and (2E)-but-2-enoic acid (Guo et al., 2010). However, these compounds are expected not to show high ORAC because they don't have hydroxyl group (Mikami *et al.*, 2009). In this context, the mechanism of tyrosinase inhibition is partly different from that of ORAC. Tyrosinase inhibition is related to whitening while ORAC is involved with antiaging (Judy, 1999), which shows antiaging indicator and is very important for cosmetic products. By the way, as for other marine algae, several kinds of them are also eaten in Okinawa, such as *Ulva pertusa*, *Gracilaria blodgetti*, *Gracilaria arcuate*, and *Codium fragile*. Unfortunately, they don't have both of high ORAC activity and tyrosinase inhibition.

Fractionation

First, HP20 in open glass column tube was dipped with Hijiki liquor. Next, methanol (MeOH) flew out through HP20. The fractionation process of Hijiki liquor was shown as Fig. 3. As a result, 58 g of dry weight in MeOH fractionation was obtained from 60 L of Hijiki liquor.

Determination of IC_{50} for tyrosinase inhibition

The IC_{50} value of Hijiki liquor was 51 μg/mL while MeOH fractionation resulted in having 3.1 μg/mL of the IC_{50} value (Fig. 3). In a word, Hijiki liquor obtained 16.5 times higher of tyrosinase inhibition after being fractionated with HP20 flowed by MeOH.

Melanin quantitation and cell viability of the human skin model

To determine the inhibitory effect of MeOH fractionation on melanogenesis, a 3D human skin model with several concentration of MeOH fractionation was cultured for 10 days. The amount of melanin on a 3D-human skin tissue model with 5 mg/mL, 10 mg/mL, and 20 mg/mL MeOH fractionation was 71 ± 23%, 69 ± 14% ($p < 0.05$), and 67 ± 4% ($p < 0.01$%), compared with control (0 mg/mL MeOH fractionation) at 100 ± 6% (Figs. 4 and 5). Therefore, 10 and 20 mg/mL of MeOH fractionation showed clearly inhibited pigmentation compared with control. In addition, to exclude the possibility that above inhibitory effects of MeOH fractionation on melanogenesis might have been caused by the inhibition of cell growth, the cell

viability of the 3D human skin model that has been topically applied with and without MeOH fractionation by MTT assay. No cytotoxicity was observed when 20 mg/mL of MeOH fractionation was applied on the tissue (142 ± 4%). Therefore, MeOH fractionation of Hijiki liquor must have decreased melanin pigmentation without affecting the cell viability.

In conclusion, raw Hijiki had the inhibitory effect of the tyrosinase activity and Hijiki liquor had the effect of reducing melanin pigmentation on 3D-human skin tissue. It is known that Hijiki has various types of phloroglucinol (Samee et al, 2009) or phlorotannins, which shows strong tyrosinase inhibition (Kang et al., 2004) and antiaging activity (Nakamura et al., 1996). Accordingly, there is a possibility that Hijiki liquor is a great material for cosmetic products, such as skin lotion, milky lotion, soap, and shampoo. Next step is to produce cosmetic products includes Hijiki liquor in a practical level.

Conclusion

Some of marine algae including popular seaweed food, Mozuku (*Cladosiphon okamuranus* Tokida) and Hijiki (*Sargassum fusiforme*), grown in Okinawa had high oxygen radical absorbance capacity and tyrosinase inhibition. By-product of Hijiki product, Hijiki liqour had the effect of decreasing melanin pigmentation and no cytotoxicity in a three-dimensional human skin model. Accordingly, there is a high possibility that Hijiki liquor is a great material for cosmetic products.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

I agree with it.

Availability of data and materials

Not applicable.

Competing interests

Not applicable.

Funding

Not applicable.

Authors' contributions

Research and analysis.

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Authors' information (optional)

Nothing special.

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Table

Table 1

Species	Order	Sampling place	H-ORAC (µmol TE/g)	L-ORAC (µmol TE/g)	Total ORAC (µmol TE/g)	Tyrosinase inhibition (%)
Padina minor	Brown algae	Onna	299.6	25	324.6	89.2
Halimeda incrassata	Green algae	Uruma	190.9	10.5	201.4	-2.8
Caulerpa cupressoides	Green algae	Uruma	131.6	32.1	163.7	2.5
Caulerpa serrulata	Green algae	Onna	56.1	87.5	143.6	3.3
Sargassum fusiforme	Brown algae	Yonabaru	124.4	7.6	132	61.0
Turbinaria ornata	Brown algae	Ishigaki	45.5	80.7	126.2	8.8
Acanthophora spicifera	Red algae	Onna	115.9	3.8	119.7	1.6
Cladosiphon okamuranus Tokida	Brown algae	Onna	103.1	9.2	112.3	78.2
Dictyopteris latiuscula	Brown algae	Iriomote	49.3	55.9	105.2	99.4
Hydroclathrus clathratus	Brown algae	Onna	72.5	18.2	90.7	-2.3
Nemacystus decipiens	Brown algae	Onna	44.6	18.5	63.1	19.7
Hormophysa cuneiformis	Brown algae	Onna	47.9	7	54.9	96.5
Halimeda macroloba	Green algae	Onna	33.3	10.5	43.8	9.2
Chnoospora implexa	Brown algae	Onna	38.2	3.1	41.3	12.4
Ceratodictyon spongiosum	Red algae	Onna	27.4	3.5	30.9	23.9
Digenea simplex	Red algae	Onna	25.4	2.9	28.3	19.7
Ulva pertusa	Green algae	Uruma	23.7	1.1	24.8	6.5
Halymenia floresia	Red algae	Iriomote	21.5	1.5	23	22.2
Gracilaria blodgetti	Red algae	Uruma	21.1	1.4	22.5	17.4
Betaphycus gelatinus	Red algae	Iriomote	19.3	1.7	21	-0.2
Gracilaria arcuata	Red algae	Ishigaki	17.1	1.3	18.4	-0.7
Asparagopsis taxiformis	Red algae	Iriomote	12.4	3.4	15.8	3.1
Hydropuntia edulis	Red algae	Ishigaki	14.7	1	15.7	1.6
Codium fragile	Green algae	Uruma	11	4.3	15.3	0.3
Hydropuntia eucheumatoides	Red algae	Ishigaki	13	0.6	13.6	7.0
Codium intricatum	Green algae	Uruma	8.2	5	13.2	11.9
Gracilaria arcuata	Red algae	Ishigaki	12.1	0.9	13	9.3
Chlorodesmis fastigiata	Green algae	Onna	8.2	0.9	9.1	23.7
Hypnea charoides	Red algae	Iriomote	6.8	1.9	8.7	14.2
Helminthocladia australis	Red algae	Uruma	6.6	1.3	7.9	-1.7

Figures

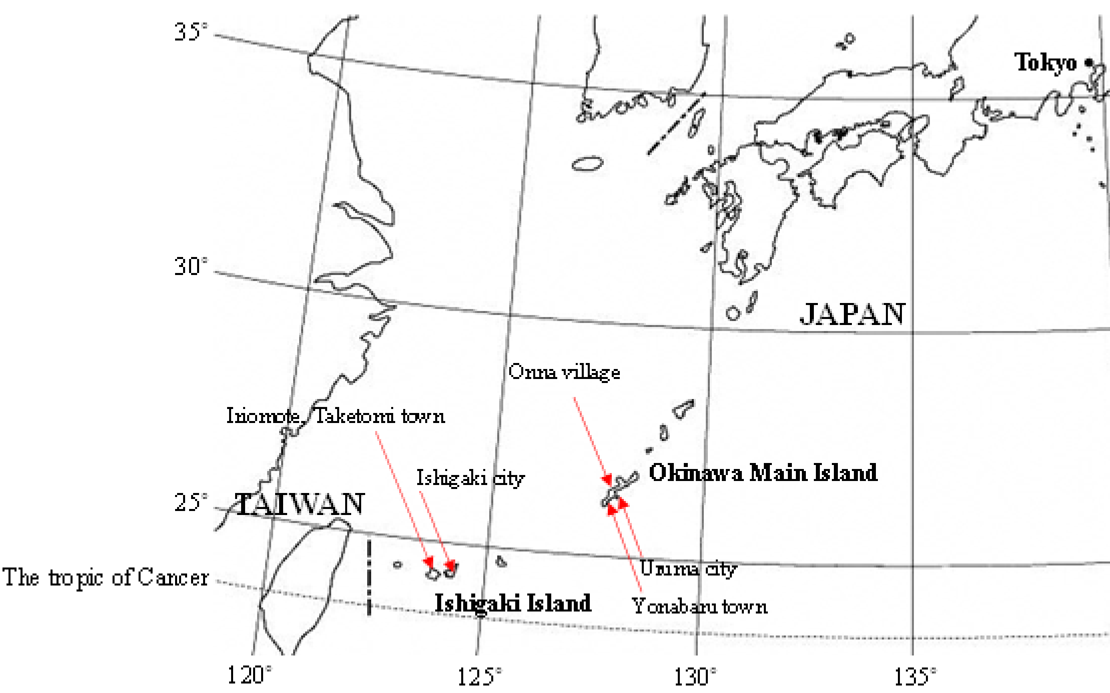

Figure 1

Map of Okinawa indicating where seaweeds were obtained. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

View of Hijiki and Hijiki liquor

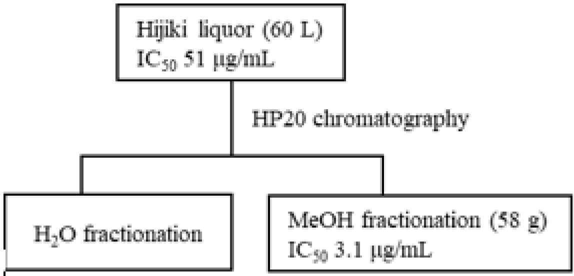

Figure 3

The fractionation process of Hijiki liquor

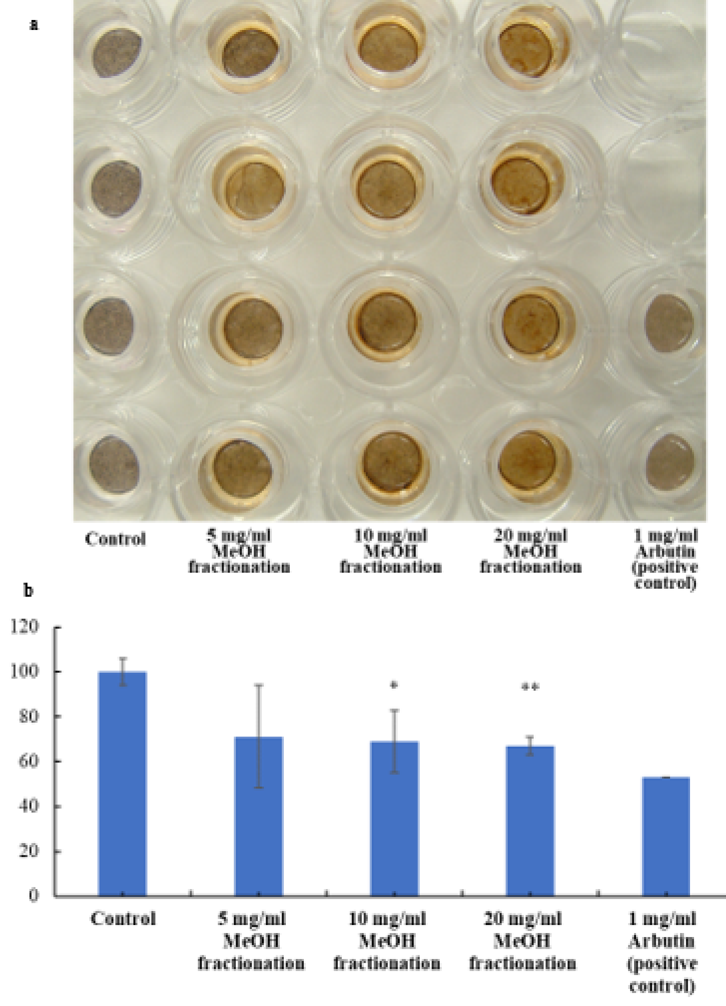

Figure 4

MeOH fractionation prevented pigmentation of melanin in a three-dimensional cultured human skin model

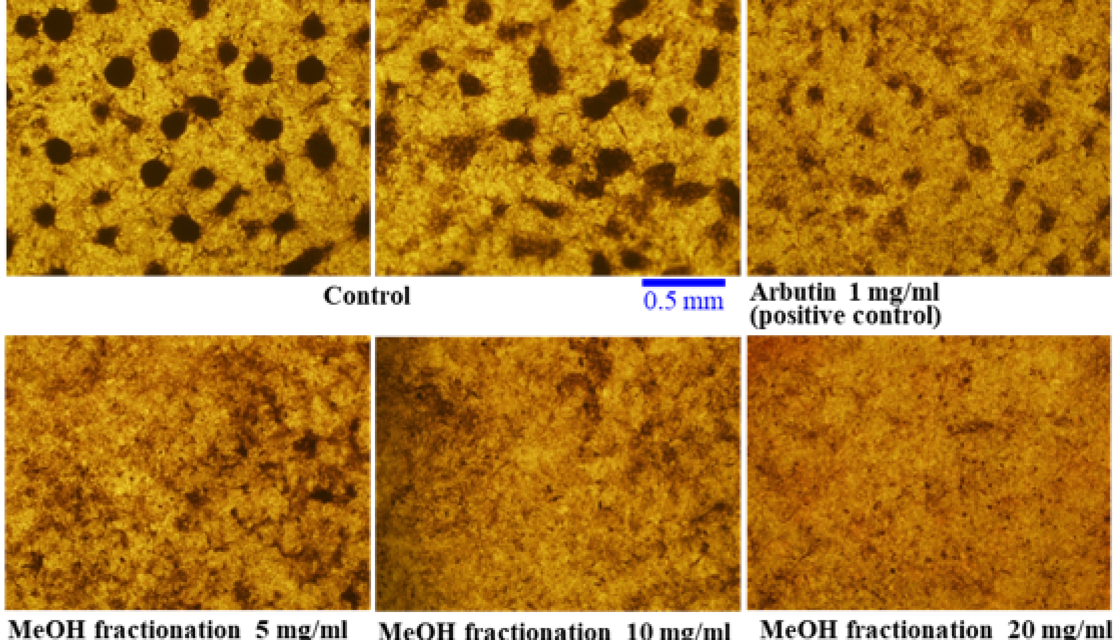

Figure 5

Microscope views of a three-dimensional cultured human skin model The tissue was cultured for 13 days and refed with 0.1 mL of the sample solution and fresh LLMM every 3 days.

Supplementary Files

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