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COSMETIC POTENTIAL OF MARINE ALGAE SUCH AS HIJIKI (*Sargassum fusiforme*) GROWN IN THE OCEAN OF OKINAWA, JAPAN

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Abstract

The ocean surrounding Okinawa has various species of marine algae that can serve as raw materials for cosmetic products because of its high concentration in phenolic compounds potentially rich in antioxidant activities. Thus, there is a need to create Okinawa's unique brands for cosmetic products using marine algae grown in the ocean of Okinawa. Some of these marine algae, including the popular seaweed food, Mozuku (*Cladosiphon okamuranus* Tokida) and Hijiki (*Sargassum fusiforme*), have been reported to possess high ORAC and tyrosinase inhibition. In food processing of Hijiki, there is a boiling step needed. This resulting Hijiki liquor is wasted, approximately up to 15 tons per year. To take advantage of this residue, the development of cosmetic products derived from this Hijiki liquor should be considered. The resulting Hijiki liquor fractionated by methanol elution using HP20 decreased melanin pigmentation, and no cytotoxicity in a three-dimensional human skin model has been observed. Therefore, there is a high possibility that Hijiki liquor is a great material for cosmetic products or ingredients.

INTRODUCTION

Okinawa is located in the southernmost prefecture of Japan and is a popular beach resort in Japan. In 2018, more than 10 million foreign tourists visited Okinawa, the highest number recorded in any year. Okinawa Prefecture consists of 160 large and small islands extending southwest from Kyushu to Taiwan. Okinawa also has an independent and unique environment surrounded by the ocean. Furthermore, Okinawa has coral reefs and crystal-clear blue seas, letting many kinds of marine algae grow there. Marine algae therein can thus be used as raw materials for cosmetic or healthy food products. One of the 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 the harmful effects of oxidative reactions on health [1]. Thus, these marine algae can be used to create Okinawa's unique brands that attract tourists. Mozuku (*Cladosiphon okamuranus* Tokida) is an Okinawan seaweed food that contains fucoxanthin, a carotenoid known for its many kinds of health benefits [2-4]. In addition, Hijiki (*Sargassum fusiforme*) grown in Okinawa is a popular food known as "Shimahijiki" that means Island Hijiki because of its unique characteristics i.e. a thicker stalk, thus making it more crunchy than those grown outside Okinawa and rich in phenolic compounds, such as phloroglucinol [5]. A boiling step is needed in the making of Shimahijiki. The process results in the annual discharge of 15 tons of cooking water (liquor). This water residue potentially contains phytochemicals with anti-aging or whitening properties and thus the development of cosmetic product derived from Hijiki liquor is warranted. In the present study, we analyzed the ORAC and tyrosinase inhibition of some kinds of seaweeds obtained in the Okinawa Ocean and investigated the melanin-decreasing effect of this Hijiki liquor on a three-dimensional human skin model.

MATERIALS

Seaweed Sample Collection

Seaweed samples of at least 200 g were collected along the coast in five areas (Table 1, Figure 1). These were washed thoroughly and kept frozen until required for freeze-drying. Hijiki was boiled using water in a food processing factory in Yonabaru. The resulting boiled water (Hijiki liquor, see Figure 2) was used for the melanin quantification on the human skin model.

Table 1: Sampling sites and characteristics of seaweed samples collected

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

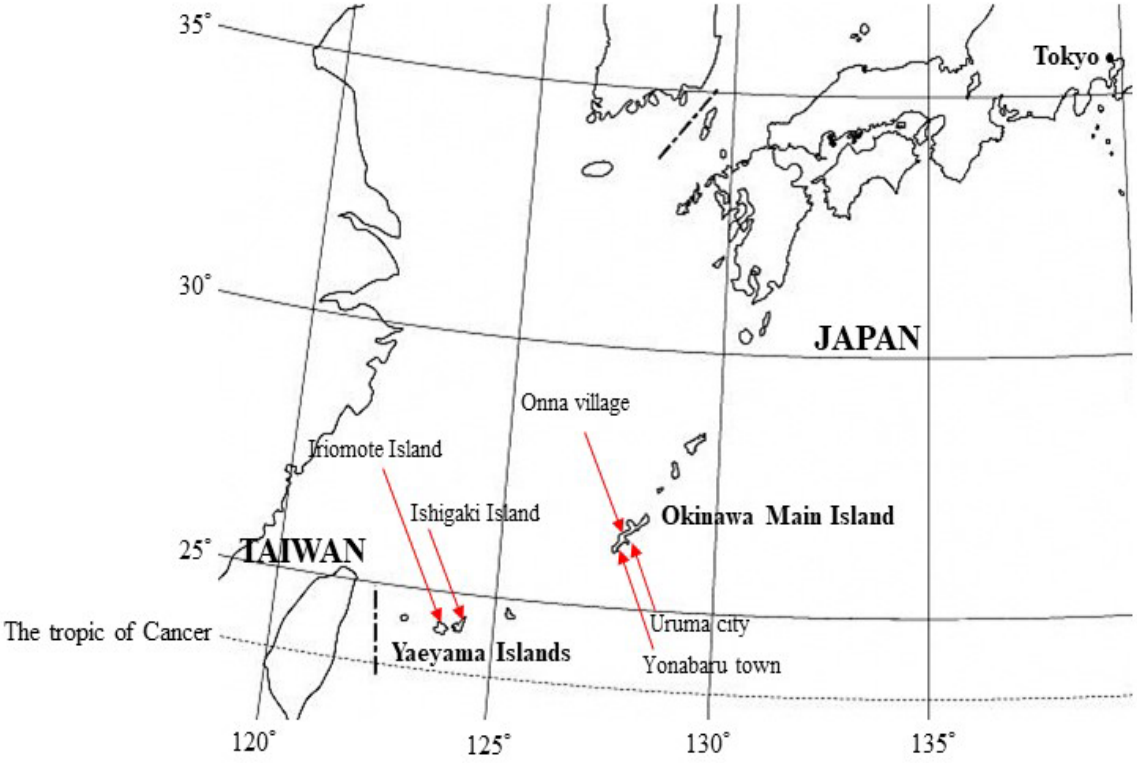

Figure 1: Map of Okinawa, indicating where seaweeds were obtained

Figure 2: View of the Hijiki algae, and its resultant Hijiki liquor

METHODS

Seaweed Powder

Collected seaweed samples were freeze-dried and then ground to powder form using a grinder (MF10 basic, IKA, Staufen, Germany). Powdered seaweed samples were then stirred enough until they reached a uniform consistency.

Extraction for ORAC Assay

The extraction process was achieved according to a modified previous protocol described by Mikami et al. (2009) [6]. Seaweed powder (100 mg) was transferred into a 10 mL screw-cap tube containing 8 mL of hexane/dichloromethane (1:1, v/v) and subjected to overnight shaking using an orbital shaker. The mixture was then centrifuged (2,000*g*, 10 min) and the supernatant

collected. The precipitate was mixed with 2 mL of hexane/dichloromethane and was vortexed for 30 seconds twice prior to centrifugation (2,000*g*, 10 min). Both hexane/dichloromethane fractions were pooled and then dried using nitrogen gas. The residue was dissolved in 0.4 mL acetone and was used for the lipophilic ORAC (L-ORAC) assay after appropriate dilution with acetone solvent. The acetone was removed using nitrogen gas and the residue was allowed to properly dissolve 4 mL of methanol/water/acetic acid (70:29.5:0.5, v/v/v) by overnight shaking in an orbital shaker. The solution was then centrifuged (2,000*g*, 10 min) and the supernatant was used for the hydrophilic ORAC (H-ORAC) assay.

ORAC Assay

ORAC value was evaluated as previously described [6]. Briefly, 110.7 nmol/L fluorescein sodium salt (SIGMA-ALDRICH, USA) and 31.7 mmol/L AAPH 2,2-azobis (2-amidinopropane) solutions were prepared in 75 mmol/L phosphate buffer solution (pH 7.4). After dilution with an assay buffer, the previously prepared sample solution was then used for the ORAC assay. The diluted samples and fluorescein solutions were also applied to the microplate. Fluorescence (excitation, 485 nm and emission, 528 nm) was recorded for the first time after a 10 min incubation period at 37℃ in a Multi-Detection Microplate Reader SH-9000 (CORONA, Ibaraki, Japan), equipped with a temperature-controlled incubation chamber, after which the microplate was taken out of the microplate reader and the AAPH solution was added into the microplate followed by placement in the microplate reader. 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 µmol averages of Trolox Equivalents (TE) per 100 g of the sample (µmol TE/g).

Tyrosinase Inhibition

Inhibitory activities of seaweed samples on mushroom tyrosinase activity were evaluated on the basis of the method [7] 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 pre-incubation at 23℃ for 3 min, 50µL of 2.5 mM L-DOPA was added to the 96-well plate. Each well's optical density at 490 nm was measured using a microplate reader (model 550, Bio-Rad Laboratories, Inc., Richmond, CA). After incubation at 23℃ for 10 min, the increase in the optical density at 490 nm was measured. The percentage inhibition of tyrosinase was also calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (A - B) / C] \times 100$$

Where A is the optical density at 490 nm with the 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 and without test sample. Three measurements of duplicate data were expressed as average %.

Fractionation

Hijiki liquor was fractionated using an open glass column tube (80 mmI.D. × 300 mm) filled with DIAION HP20 (HP20) (Mitsubishi Chemical Co. Ltd. Tokyo, Japan), and special grade methanol (MeOH) (NACALAI TESQUE, INC. Kyoto, Japan) was used as eluent to move the compounds through the column. HP20 is a styrene-divinylbenzene synthetic adsorbent used for polyphenol isolation [8].

Determination of IC_{50} for Tyrosinase Inhibition

The extent of inhibition upon Hijiki liquor addition is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC_{50}), which was extrapolated from the curve constructed from percentage activity vs. various concentrations of the sample tested.

Culture for the Human Skin Model

The three-dimensional (3D) human skin model (MEL-300-B kit; Kurabo) was placed on a 6-well plate with 0.9 mL of the medium and incubated at 37℃ for 1 h in a humidified atmosphere supplemented with 5% CO_2. 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 containing 5, 10, and 20 mg/mL of MeOH fractionation dissolved in 12.5% glycerol was applied to the surface of the tissue on a model cup (n = 4 except positive control, n = 2). The tissue was then cultured for 10 d with fresh LLMM medium change containing 0.1 mL of the sample solution every two days. Melanin production and viability of tissue cells were measured at the end of the 10 d culture period.

Melanin Quantitation for the Human Skin Model

The melanin production by tissue cells of the three-dimensional (3D) cultured human skin model was measured using a minor modification to the method [9]. The skin model was placed on a 24-well plate and the tissue surface was rinsed three times with Dulbecco PBS. The tissue was then treated with 0.45 mL of 10 mM Tris-HCl buffer, (pH 6.8, which contained 1% SDS and 0.05 mM EDTA), and was incubated at room temperature for 3 h in an airtight container containing 20 µL of 5 mg/mL proteinase K. The tissue was then transferred from the

model cup to a 1.5 mL tube and was re-incubated at 45℃ 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 then rinsed 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 rinsed tissue was subsequently fixed in 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 then mixed with 0.5 M sodium carbonate (50 µL) and 30% hydrogen peroxide (10 µL), incubated at 80℃ for 30 min, and then allowed to cool. The lipid from the tissue was removed by adding 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 then measured at 405 nm on a 96-well plate using 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 using the MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] dye reduction assay. At the end of the culture, the model cup was rinsed three times with Dulbecco PBS. The skin model was then placed on a 24-well plate, after which 300 µL of the MTT solution (MTT-100 kit, Kurabo) was added, and the tissue was incubated at 37℃ for 3 h in a humidified atmosphere, which contained 5% CO_2. After incubation, the tissue was washed with Dulbecco-PBS. The skin model was then placed on another 24-well plate, after which 2 mL of the 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 also 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, a brown alga, had the highest H-ORAC (299.6 µmol TE/g) and total ORAC (324.6 µmol TE/g) values among seaweeds obtained (Table 1). Furthermore, *Halimeda incrassata*, a green alga, had the second-highest H-ORAC (190.9 µmol TE/g) and total ORAC (201.4 µmol TE/g) values. Among the 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*, a green alga, had the highest value (87.5 µmol TE/g), followed by *Turbinaria ornata* (80.7 µmol TE/g) and *Dictyopteris latiuscula* (55.9 µmol TE/g), which are both brown algae. 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 [10] while H-ORAC values correlated well with the polyphenol contents [11]. As a result, these three species can be used as vitamin E and other sources 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 foods in Okinawa, had high H-ORAC values (124.4 µmol TE/g and 103.1 µmol TE/g). Also, it is interesting that Hijiki (*Sargassum fusiforme*) and Mozuku (*Cladosiphon okamuranus* Tokida) had inhibitory effects on tyrosinase activity (61.0% and 78.2%). Thus, they are expected to be nutritious functional foods with high potential in cosmetic production.

In tyrosinase inhibition, *Dictyopteris latiuscula* had 99.4% tyrosinase inhibition, *Hormophysa cuneiformis*, 96.5%, and *Padina minor* 89.2%. Although these brown algae possess tyrosinase inhibition in abundance, they are not known to be consumed. Thus, there is a need to conduct further cytotoxicity tests to verify their safe use in cosmetic products. Although green algae *Halimeda incrassate*, *Caulerpa cupressoides*, and *Caulerpa serrulate* had high total ORAC values, they do not have tyrosinase inhibitory functions. The hydrophobic p-alkyl group inhibits the mushroom tyrosinase [12], including unsaturated fatty acids, such as (2E, 4E)-hexa-2,4-dienoic acid and (2E)-but-2-enoic acid [13]. However, these compounds are expected not to show high ORAC because they do not have a hydroxyl group [6]. In this context, the mechanism of tyrosinase inhibition was partly different from that of ORAC. The tyrosinase inhibition was related to whitening, while ORAC was involved in anti-aging [14], crucial in cosmetic production. Several types of marine algae are eaten in Okinawa, such as *Ulva pertusa*, *Gracilaria blodgetti*, *Gracilaria arcuate*, and *Codium fragile* but they lack both high ORAC activity and tyrosinase inhibitory effect.

Fractionation of Hijiki Liquor

The fractionation process of Hijiki liquor was carried out as shown in a flow diagram in Figure 3. We obtained 58 g of dry weight in MeOH fractionation from 60 L of the Hijiki liquor.

Tyrosinase Inhibition

The IC_{50} value of Hijiki liquor tyrosinase inhibition was 51 µg/mL while MeOH fractionation of the liquor decreased the IC_{50} value to 3.1 µg/mL. Thus, the fractionation of Hijiki liquor with HP20 resulted in 16.5 times increase in tyrosinase inhibition.

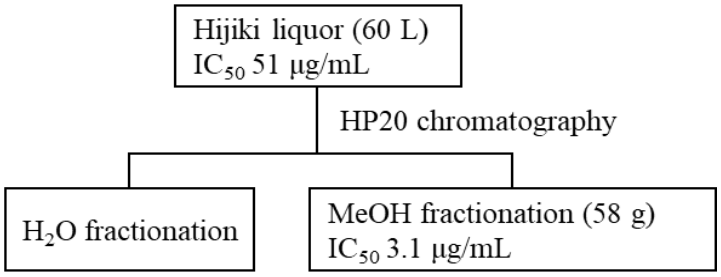

Figure 3: The fractionation process involved in Hijiki liquor production

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 was cultured for 10 d in the presence of several concentrations of MeOH fractionations. The amount of melanin on the 3D-human skin tissue model was 71% ± 23%, 69% ± 14% ($p < 0.05$), and 67% ± 4% ($p < 0.01$) when 5, 10, and 20 mg/mL of MeOH fractionation was used, compared with the control (0 mg/mL MeOH fractionation) at 100% ± 6% (Figure 4 and 5). A clear pigmentation inhibition compared to control was observed when 10 and 20 mg/mL of MeOH fractionation were used. The cell viability of the 3D- human skin model, was used to carry out experiments to exclude the possibility that the inhibitory effects of MeOH fractionation on melanogenesis are caused by the inhibition of cell growth. No cytotoxicity was observed when 20 mg/mL of MeOH fractionation was applied to the tissue (142% ± 4%, Table 2). Therefore, the MeOH fractionation of the Hijiki liquor decreased melanin pigmentation without affecting cell viability.

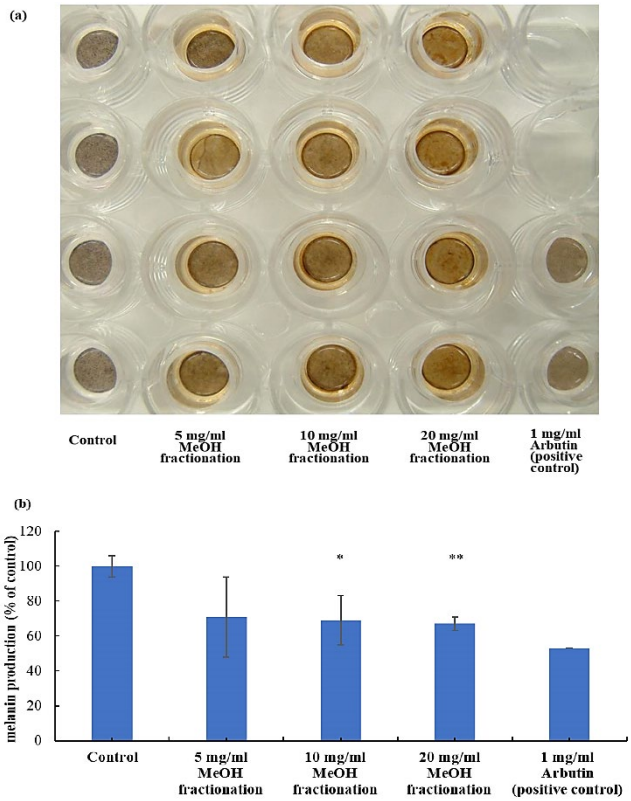

Figure 4: Hijiki MeOH fractionation prevented pigmentation of melanin in a three-dimensional cultured human skin model. a) Macroscopic view of cultured tissues; b) Melanin of the tissues was quantified. Bars represent the mean ± SD (n = 4). ** represents significant differences from the control at $p < 0.01$; * represents a significant difference from the control at $p < 0.05$.

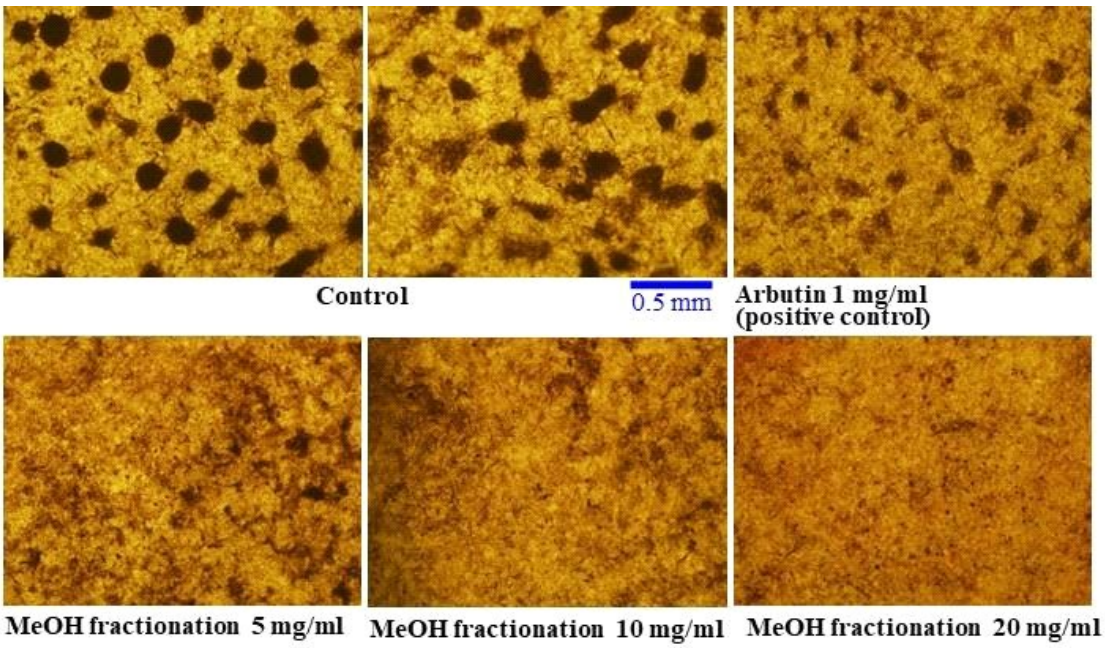

Figure 5: Microscopic views of a three-dimensional cultured human skin model. The tissue was cultured for 13 d, and re-fed with 0.1 mL of the sample solution and fresh LLMM every two days.

Table 2: Cytotoxicity of 20 mg/mL of MeOH fractionation on a three-dimensional human skin model[a]

Control	100 ± 3
MeOH fractionation 20 mg/mL	142 ± 4

% of control (mean ± standard deviation) (n=2) at OD = 570 nm
[a]Tissues were cultured for 13 days.

In conclusion, raw Hijiki had inhibitory effects on tyrosinase activity, while the Hijiki liquor reduced melanin pigmentation on 3D-human skin tissue. Hijiki also has various types of phloroglucinol [5] or phlorotannins, which show strong tyrosinase inhibition [15] and anti-aging activity [16]. Thus, Hijiki liquor has great potential to be used as food products, nutritional supplements and raw material for cosmetic and skincare products such as skin lotion, milky lotion, soap, and shampoo.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this manuscript.

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