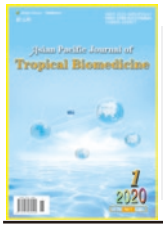

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Antioxidant and anti-melanogenic activities of ultrasonic extract from *Stichopus japonicus*

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ABSTRACT

Objective: To investigate the antioxidant and anti-melanogenesis activities of an ultrasonic extract of red sea cucumber, *Stichopus japonicus*, collected from Jeju Island.

Methods: Antioxidant activity experiments were assessed by an electron spin resonance system and a cellular model of immortalized human keratinocytes (HaCaT) to determine its radical scavenging activity and protective effects against 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress. Anti-melanogenic activity of the ultrasonic extract of red sea cucumber was also examined using the melanoma cell model B16F10 and mushroom tyrosinase. Following the induction by α-melanocyte-stimulating hormone, the effects of the ultrasonic extract of red sea cucumber on intracellular tyrosinase activity, melanin content and the melanogenic protein expression of microphthalmia-associated transcription factor, tyrosinase, and tyrosinase-related proteins (TRP-1, and TRP-2) were examined.

Results: The ultrasonic extract of red sea cucumber significantly scavenged 2,2-diphenyl-1-picrylhydrazyl and alkyl radicals [IC_{50}: (0.924±0.035) and (0.327±0.006) mg/mL, respectively], as well as showed a protective effect against oxidative stress and attenuated generation of intracellular reactive oxygen species on AAPH-induced HaCaT cells, with no cytotoxicity (12.5-400 μg/mL). The ultrasonic extract of red sea cucumber also exhibited a tyrosinase inhibitory effect [IC_{50}: (2.750±0.006) mg/mL]. On α-melanocyte-stimulating hormone-stimulated B16F10 melanoma cells, the ultrasonic extract of red sea cucumber (25-200 μg/mL) significantly inhibited not only melanin synthesis and tyrosinase activity, but also protein expressions of microphthalmia-associated transcriptional factor, tyrosinase, TRP-1, and TRP-2.

Conclusions: The ultrasonic extract of red sea cucumber shows antioxidant and anti-melanogenic potential and may be a natural candidate for anti-aging as well as a whitening agent in the cosmeceuticals industry.

KEYWORDS: Red sea cucumber (*Stichopus japonicus*); Ultrasonic extract; Antioxidant activity; Anti-melanogenesis; Cosmeceuticals

1. Introduction

Aging is an inevitable process in human life and all organs naturally degrade with the passage of time, including skin[1]. Skin aging is described as one of two types, chronological or intrinsic aging and extrinsic aging. Intrinsic aging undergoes the natural aging process which normally displays around 30-40 years in humans, whereas extrinsic aging refers to premature aging due to environmental factors such as ultraviolet radiation, chemicals, hormones and/or lifestyle[2,3]. Owing to several unpleasant visible signs such as wrinkles, dryness, irregular pigmentation, freckles, and leathery appearance, aging-skin has been considered a targetable therapeutic issue[1-3].

Reactive oxygen species (ROS) are unstable free radicals, found in many cellular mechanisms, which lead to oxidative stress and DNA damage, as well as cell death. In addition, various age-related enzymatic systems including hyaluronidase, tyrosinase, collagenase, and elastase are also activated during the ROS accumulation

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process[1,3,4]. Antioxidants can counteract unpaired free radicals and in doing so defend from the harmful phenomena initiated by excess oxidative stress. Those superb protective mechanisms can facilitate delays to the premature aging of skin[4].

Unhealthy or aged skin appearance can occur due to pigmentation disturbances caused by the overproduction of melanin such as hyperpigmentation, melasma, ephelides, lentigo, age spots, and post-inflammatory pigmentation[5]. Discoloration events along with the aesthetic concerns of certain cultural preferences particularly Asian, have resulted in melanin inhibitors or whitening agents becoming one of the most in-demand products in the cosmeceutical field[6,7]. In fact, tyrosinase inhibitors such as hydroquinones, arbutin, azelaic acid, and kojic acid are currently available in the cosmeceutical market, however, several reports have mentioned adverse effects or defective design in clinical trials[7,8]. Thus, efforts to uncover novel melanogenesis inhibitors with both powerful efficacy and safe potential have continued because of these inadequacies[9].

An extended process and the requirement for large amounts of organic solvents which are harmful to the environment and living-things, were reported as drawbacks to conventional Soxhlet extraction[10,11]. Recently, ultrasonication has been used on marine organisms as an extraction method to overcome the difficulties of traditional extractions[10,12]. Ultrasonic extraction (UE) is an inexpensive method which has several advantages over the traditional method, such as providing a less time-consuming process, requiring less solvent, in conjunction with the greater extraction yield. Moreover, UE only requires a minor amount of energy to achieve cell disruption by ultrasound waves, which then facilitates better penetration of solvent to the tissue and allows for easier release of intracellular compounds to the bulk solvent. Hence, UE has been widely employed for the extraction of various natural and marine-derived products[10-13].

Marine organisms are a massive available renewable resource that comprises a variety of biological and chemical compounds with genetic diversity[14]. Therefore, marine-derived compounds display abundant bioactivity and physiological inhibitions which are beneficial to various industries including the food, drug and cosmetics industries. Owing to the stability and safeness of natural products which are often superior to synthetic-derived molecules, marine organisms are an attractive resource to discover new bioactive substances[14,15].

Sea cucumber is a marine-derived organism widely used as a traditional remedy among East Asian countries especially China, Japan and Korea[14]. Sea cucumber is becoming a highly consumed and costly seafood because of its broad benefits[16]. Aside from the utilization of nourishing supplements, there are an abundance of bioactive properties of sea cucumber including anti-tumor, immunoregulatory, anti-coagulant, anti-thrombin, anti-viral and wound healing properties that have been reported[9,16-18]. Sea cucumber, also known as *Stichopus japonicus* (*S. japonicus*), is normally differentiated by color in three different groups; black, green and red. Among them, red sea cucumber (RSC) is remarkable for its uniqueness of habitat, pigment compositions and nutrient components, compared with the other color types[18]. To date, no studies have extensively investigated the bioactivity of ultrasonic extracts of RSC (*S. japonicus*), in terms of antioxidant and anti-melanogenic effects. Therefore, in this present study, RSC collected from Jeju Island of South Korea was prepared by UE and evaluated for its antioxidant and anti-melanogenesis potential.

2. Materials and methods

2.1. Materials

Jeju RSC tissue dried by far infrared radiation dryer was kindly provided by the Jeju International Marine Science Research & Education Center of the Korea Institute of Ocean Science & Technology (KIOST). All other chemicals and solvent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium, penicillin/streptomycin, and fetal bovine serum were purchased from Gibco BRL (Life Technologies, Burlington, ON, Canada). The BCA kit and ECL reagents were purchased from Thermo Fisher Scientific (Rockford. lL, USA). Antibodies against tyrosinase, tyrosinase-related protein-1 and protein-2 (TRP-1 and TRP-2), and microphthalmia-associated transcription factor (MITF) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit IgG was purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Preparation of red sea cucumber ultrasonic extracts (RSC-UE)

Red sea cucumber (voucher number: KIOST_RSC_1) was collected from the coast of the Jeju Island, Korea, and identified by Jeju International Marine Science Research & Education Center of the Korea Institute of Ocean Science & Technology. The experiment was carried out in an ultrasonic extraction reactor, equipped with a transducer at the base of the jug, operating at a frequency of 20 kHz with ultrasound electric power of 1 200 W. The ground RSC powder (1 kg) was mixed with 20 L of water and placed in an ultrasonic extraction reactor for 3.5 h at 8 ℃. The extracts were centrifuged at 3 500 rpm for 20 min at 4 ℃ and filtered through Whatman filter paper to remove the residue. The RSC-UE yield after freeze-drying was 450 g/1 kg of powdered RSC.

2.3. Measurement of free radical scavenging activity

Free radical scavenging activities determining the levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and alkyl radicals were performed by electron spin resonance (ESR) measurements at room temperature (JES-FA machine, JEOL, Tokyo, Japan).

2.3.1. DPPH radical scavenging activity

DPPH radical scavenging activity was measured with the method described by Nanjo *et al*[19]. A distilled water solution of 60 μL of the test sample (distilled water as a control) was added to 60 μL DPPH (60 μM) in methanol solvent, and the sample was mixed vigorously for 2

min. The solution was transferred to a capillary tube. The experimental conditions were as follows: power, 1 mW; amplitude, 1×1 000; modulation width, 0.8 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s.

2.3.2. Alkyl radical scavenging activity

Alkyl radicals were measured according to the method described by Hiramoto *et al*[20]. Briefly, 20 μL of 40 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and 20 μL of 40 mM α-(4-pyridyl-1-oxide)-N-tert-butylnitrone were mixed with 20 μL of phosphate-buffered-saline (PBS) and 20 μL of the test sample. The mixture was incubated for 30 min at 37 ℃ in a water bath and then transferred to the capillary tube. The experimental conditions were as follows: power, 10 mW; amplitude, 1×1 000; modulation width, 0.2 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s.

2.4. Cell culture

HaCaT and B16F10 cells were obtained from the Korean cell line bank. Both cell lines were routinely grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin, 10 μg/mL streptomycin in a humidified incubator with 5% carbon dioxide, at 37 ℃.

2.5. Cell viability assay

The HaCaT cells were seeded at a density of 1×10^5 cells/mL in a 96 well plate for 24 h and then treated with RSC-UE at 12.5, 25, 50, 100, 200, 400, or 800 μg/mL for 24 h. B16F10 cells were seeded in 24-well plates at 3×10^4 cells/mL and incubated for 24 h. Cells were treated with RSC-UE at 25, 50, 100, 200, 400 μg/mL for 72 h. To test the effects of RSC-UE on AAPH-induced oxidative stress in HaCaT cells, the cells were treated with 10 mM AAPH and cultured in complete culture medium with 12.5, 25, 50, 100, 200, 400 μg/mL RSC-UE for 24 h. Cell viability was determined using a colorimetric MTT assay. MTT stock solution (2 mg/mL in PBS) was added to each well. After 4 h of incubation, medium was then discarded from each well and the cells were dissolved with DMSO. The absorbance was measured at 540 nm using a microplate reader (Sunrise TW, Tecan Trading AG, Männedorf, Switzerland).

2.6. Measurement of intracellular ROS

Intracellular ROS production was measured by the 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay following the previous study by Yuan *et al*. with some modifications[21]. Briefly, HaCaT cells were seeded on a 96-well plate at 1×10^5 cells/mL. After 16 h, cells were treated with various concentrations of RSC-UE and then incubated at 37 ℃. After 1 h, 10 mM of AAPH was added to cells and incubated for 30 min at 37 ℃. DCF-DA (5 μg/mL) was introduced to the cells. DCF-DA fluorescence was detected at an excitation of 485 nm and an emission at 535 nm using a Perkin-Elmer LS-5B spectrofluorometer (Perkin Elmer Inc., Massachusetts, USA).

2.7. Measurement of the inhibitory effects of RES-UE on mushroom tyrosinase

The inhibition of mushroom tyrosinase was measured was using *L*-tyrosine as substrate according to the method of Heo *et al*[22]. Briefly, 10 μL of the indicated concentrations (0.5, 1, 2, and 4 mg/mL) sample solution or arbutin (1 mg/mL) as a positive control, 10 μL of the aqueous solution of mushroom tyrosinase (1 500 units/mL), 20 μL of the 1.5 mM *L*-tyrosinase and 110 μL of 100 mM sodium phosphate buffer were added to a 96 well microplate and incubated for 12 min at 37 ℃, then kept on ice for 1 min to stop the reaction. The absorbance was determined at 475 nm using a microplate reader.

2.8. Melanin content and cellular tyrosinase activity in B16F10 cells

B16F10 cells were seeded at 3×10^4 cells/mL in 6-well plates and incubated 24 h. The cells were co-treated with α-melanocyte stimulating hormone (α-MSH) and RSC-UE (0-200 μg/mL) or 100 μg/mL arbutin for 72 h. Cells were washed with ice-cold PBS and harvested using trypsin-EDTA. The cell pellet was obtained by centrifugation. The cell pellets were dissolved in 300 μL of 1 N NaOH containing 10% DMSO and incubated at 80 ℃ for 1 h. The absorbance at 490 nm was measured using a microplate reader.

In the tyrosinase activity assay, the cells were lysed in 50 mM sodium phosphate buffer (containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 12 000 *g* for 30 min at 4 ℃. After protein quantification and normalization, 80 μL of cell lysates (each sample contained the same amount of protein) were incubated in duplicate with 20 μL of 10 mM *L*-DOPA at 37 ℃ for 1 h. After incubation, dopachrome was monitored by measuring the absorbance at 475 nm using a microplate reader.

2.9. Western blot analysis

The effect of RSC-UE on the expression of melanogenesis-related proteins was detected *via* Western blot analysis. B16F10 cells seeded in a 10-cm dish for 24 h, were co-treated with α-MSH and RSC-UE (0-200 μg/mL) or 100 μg/mL arbutin for 72 h. After treatment, the cells were lysed with lysis buffer (20 mM Tris, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 10 mg/mL aprotinin, 10 mg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined by BCA™ protein assay. Samples containing equal amounts (50 μg) of protein were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, separated by electrophoresis and transferred to a nitrocellulose membrane. Membranes were then blocked with Tris-buffer containing 0.1% Tween 20 and 5% skimmed milk for 2 h and incubated with MITF, tyrosinase, TRP-1, TRP-2, and GAPDH antibodies for 16 h at 4 ℃. Membranes were incubated with secondary antibodies at 1:3 000 dilution at room temperature for 2 h. Signals were developed using a chemiluminescence (ECL) western blotting detection kit and exposed to X-ray film (Fusion Solo, Vilber

Lourmat, France) and the relative expressions of all proteins were estimated by using Image J program.

2.10. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of triplicate experiments. Statistical comparisons of the mean values were performed by analysis of variance (one-way ANOVA), followed by Duncan's multiple range test. For all analyses, a probability value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Radical scavenging activity of RSC-UE

The scavenging ability of the RSC-UE towards DPPH and alkyl free radicals by ESR is shown in Figure 1. DPPH free radical scavenging activities of RSC-UE significantly increased in a dose-dependent manner. Likewise, alkyl radical scavenging activities of RSC-UE at 0.125, 0.25, 0.5 and 1 mg/mL exhibited dose-dependent increases with (24.07±3.66), (42.11±1.07), (70.73±2.09) and (84.12±2.69)% inhibition, respectively. Furthermore, the RSC-UE showed a slightly stronger response in alkyl free radical inhibition [IC_{50}= (0.327±0.006) mg/mL] than DPPH free radical scavenging [IC_{50}= (0.924±0.035) mg/mL].

3.2. The effect of RSC-UE against AAPH-induced oxidative stress in HaCaT cells

The cytotoxicity of RSC-UE in HaCaT cells was investigated by MTT assay (Figure 2A). The results showed that the viability of cells treated with RSC-UE at 12.5-400 μg/mL had no significant difference compared with that of untreated control. Thus, concentrations of RSC-UE between 12.5-400 μg/mL were chosen for further studies. The protective effect of RSC-UE was evaluated by adding 10 mM AAPH into HaCaT cell in order to induce oxidative stress. Compared to untreated cells, cell viability was significantly diminished by approximately 50% in AAPH-stimulated cells. RSC-UE increased the survival rate of AAPH-treated cells, which was correlated with the doses of RSC-UE (12.5-400 μg/mL) (Figure 2B). In the same way, DCF-DA assay exhibited ROS generation in AAPH-induced cells that was 4-times higher than the control [(433.07±21.89)% of the control]. RSC-UE reduced ROS production in treated cells in a dose-dependent manner. Likewise, pretreatment of RSC-UE in ROS-induced cells obviously attenuated the fluorescence intensity reflecting the decreased ROS level, as shown in Figure 3.

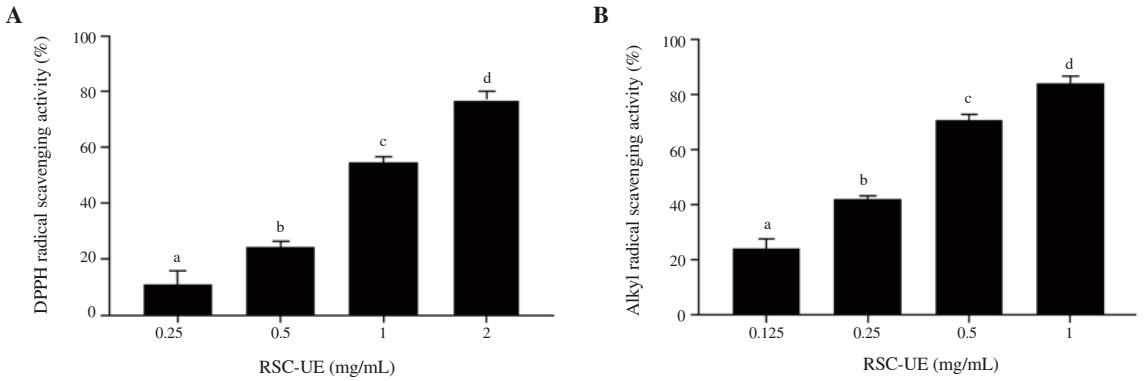

Figure 1. DPPH (A) and alkyl (B) radical scavenging activities of an ultrasonic extract of red sea cucumber (RSC-UE). IC_{50} value is the concentration of sample required for 50% inhibition. The values are expressed as mean ± SD in triplicate experiments. [a-d]Values with different letters are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test. DPPH: 2,2-diphenyl-1-picrylhydrazyl.

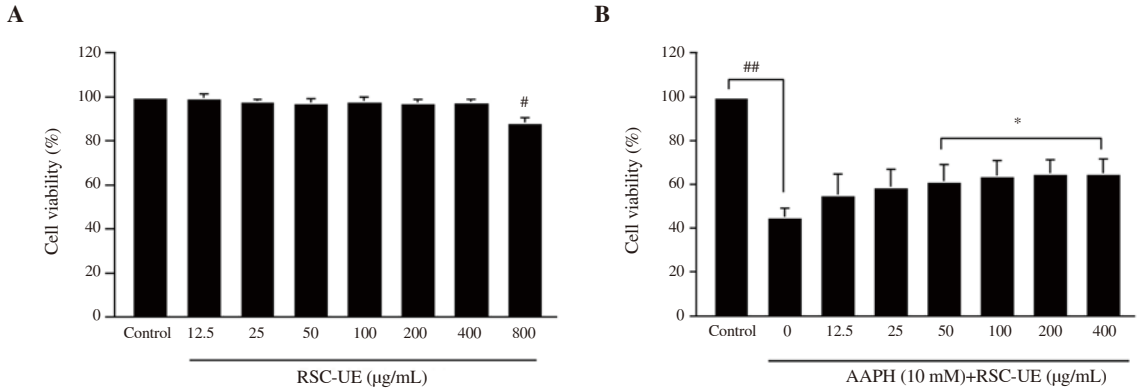

Figure 2. Cytotoxicity of RSC-UE in HaCaT cells (A) and effect of RSC-UE on cell viability in AAPH-treated HaCaT cells (B). Values are expressed as mean ± SD in triplicate experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate a significant difference compared to the control groups and $^{*}P < 0.05$ indicates a significant difference compared to the group treated with AAPH alone.

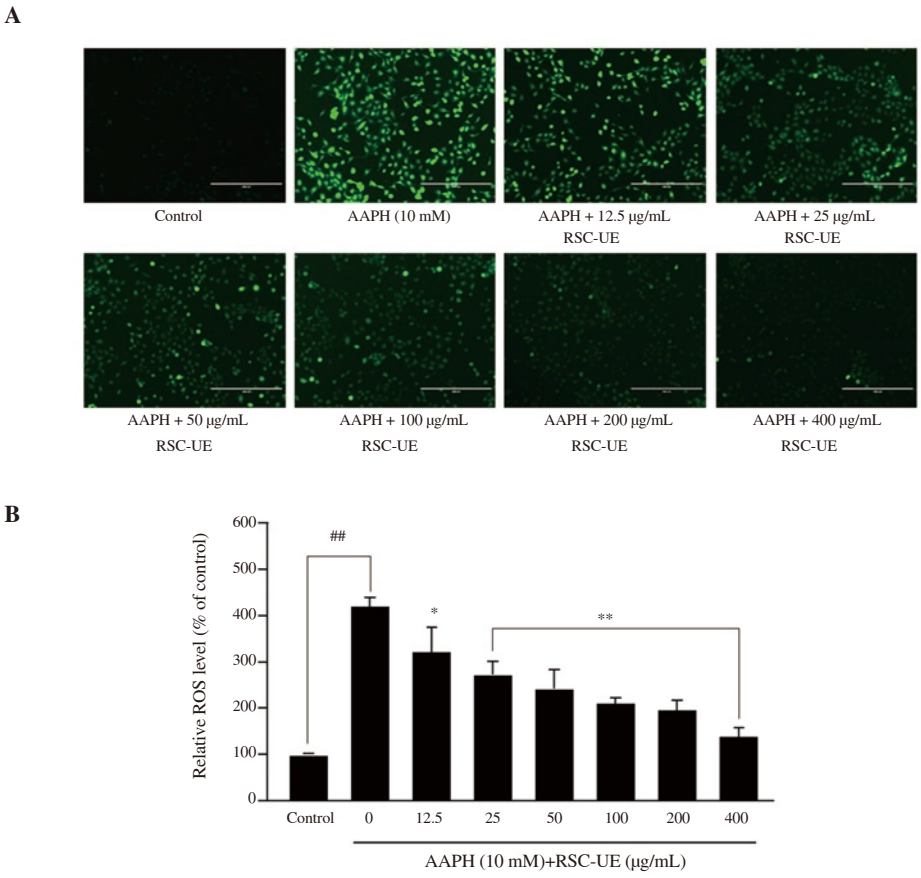

Figure 3. Effect of RSC-UE on intracellular ROS generation in AAPH-treated HaCaT cells. ROS levels were assessed by fluorescence microscopy after DCF-DA staining (A). ROS generation was assessed by the DCF-DA assay (B). Values are expressed as mean ± SD in triplicate experiments. $^{\#\#}P < 0.01$ indicates a significant difference compared to the control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference compared to only AAPH-treated groups. AAPH: 2,2'-azobis (2-amidinopropane) dihydrochloride.

3.3. Inhibitory effect of RSC-UE against mushroom tyrosinase

The tyrosinase inhibitory effect was measured by spectrophotometric assay. RSC-UE remarkably increased inhibitory effect with increasing concentration [IC_{50}: (2.750±0.006) mg/mL]. The effect of 4 mg/mL of RSC-UE was similar to that of arbutin, a commercial whitening compound, which was used as a positive control (Figure 4).

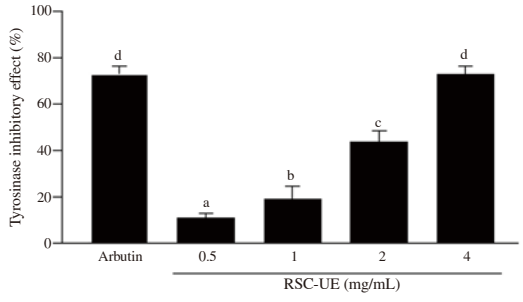

Figure 4. Inhibitory effect of RSC-UE against mushroom tyrosinase. Inhibitory effects were determined with *L*-tyrosine as the substrate and arbutin was employed as a positive control. IC_{50} value is the concentration of sample required for 50% inhibition. The values are expressed as mean ± SD in triplicate experiments. $^{a\text{-}d}$Values with different letters are significantly different at $P < 0.05$ as analyzed by Duncan's multiple range test.

3.4. Cytotoxicity of RSC-UE in B16F10 melanoma cells

We first investigated whether RSC extracts affected the viability of B16F10 cells and found there was little toxicity. Hence, based on the acceptable viabilities at concentrations of 25-200 μg/mL, this range was chosen for further examination (Figure 5).

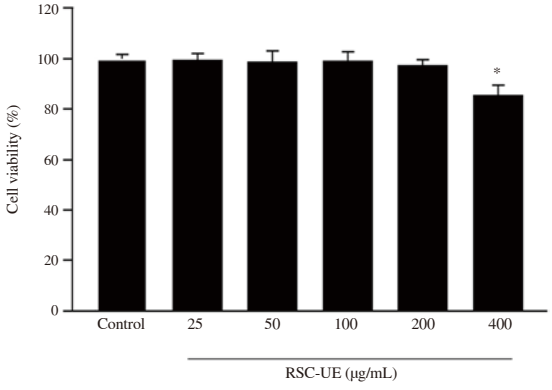

Figure 5. Cytotoxicity of RSC-UE in B16F10 melanoma cells. Values are expressed as mean ± SD in triplicate experiments. $^{*}P < 0.05$ indicates a significant difference compared to the control group.

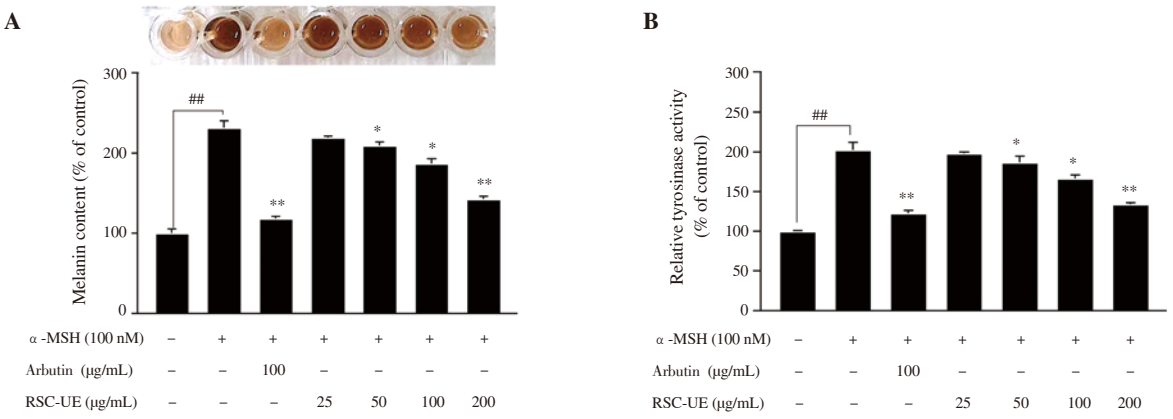

Figure 6. Effect of RSC-UE on melanin synthesis (A) and cellular tyrosinase activity (B) in B16F10 cells. Cells were exposed to 100 nM α -melanocyte stimulating hormone (α -MSH) in the presence of the indicated concentrations of extracts or 100 μg/mL arbutin. Values are expressed as mean ± SD in triplicate experiments. $^{\#\#}P < 0.01$ indicates a significant difference compared to the control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference compared to the group treated with α -MSH alone.

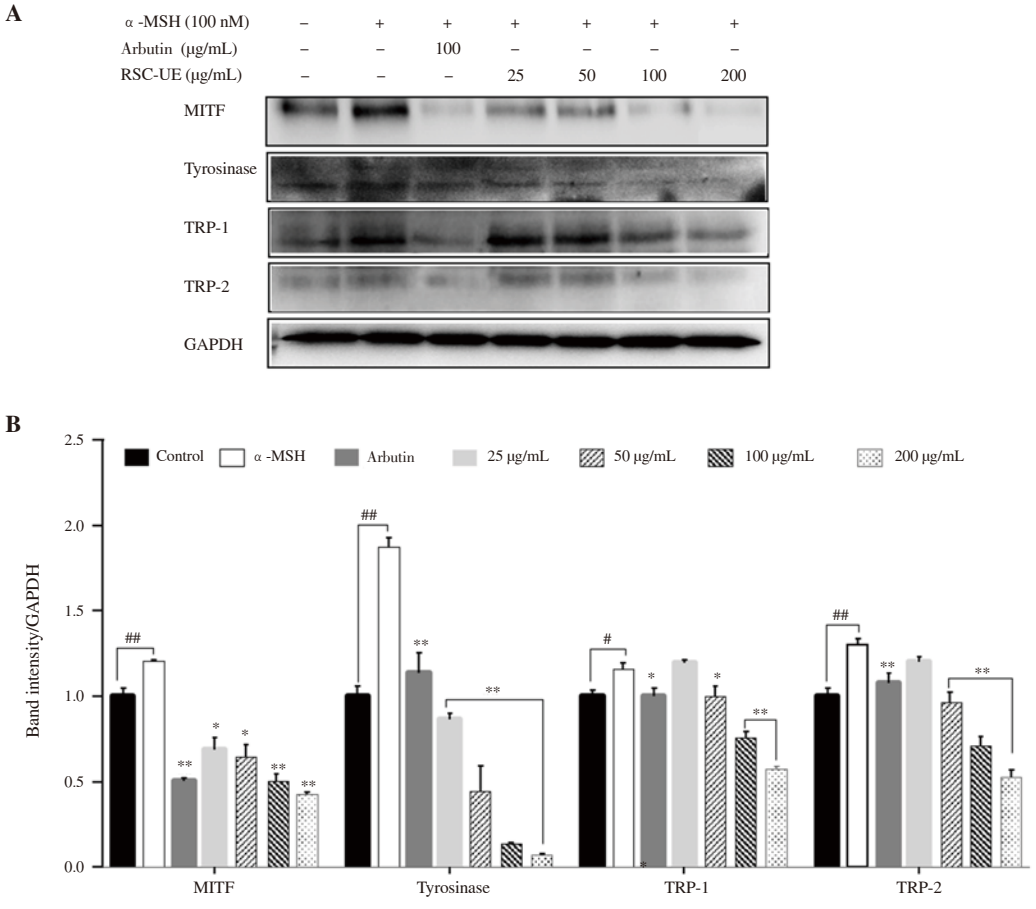

Figure 7. Effect of RSC-UE on expression of melanogenesis-related proteins in B16F10 cells. Cells were exposed to 100 nM α -MSH in the presence of the indicated concentrations of extracts or 100 μg/mL arbutin. (A) microphthalmia-associated transcription factor (MITF), tyrosinase, TRP1, and TRP2 protein expression; (B) Quantification of MITF, tyrosinase, TRP1, and TRP2 expression. The values are expressed as mean ± SD in triplicate experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate a significant difference compared to the control group and $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference compared to the group treated with α -MSH alone.

3.5. Effects of RSC-UE on cellular tyrosinase activity and melanin content in B16F10 melanoma cells

The anti-melanogenesis effects of RSC-UE were investigated by measuring tyrosinase activity and melanin formation on α-MSH-stimulated B16F10 cells. As shown in Figure 6, the results indicated that α-MSH (100 nM) accelerated tyrosinase activity and melanin production in B16F10 cells by approximately 2.2 fold compared to the control group. In the α-MSH-induced cells treated with RSC-UE at 25-200 μg/mL, we found that RSC-UE significantly inhibited tyrosinase activity as well as melanin synthesis in a dose-dependent manner compared with α-MSH treated cells alone. Arbutin, used as a positive control, possessed a greater inhibitory effect than RSC-UE at 100 μg/mL. Moreover, the darkening color of the harvested cell could be seen to change dose-dependently with RSC-UE.

3.6. Effect of RSC-UE on MITF, tyrosinase, TRP-1 and TRP-2 expressions in B16F10 melanoma cells

RSC-UE at the concentrations ranging from 25-200 μg/mL reduced enzyme expression levels (tyrosinase, TRP-1 and TRP-2) in a dose-dependent manner as shown in Figure 7. Likewise, levels of MITF were downregulated with increasing concentrations of RSC-UE. Moreover, α-MSH stimulated cells ended up with higher expressions for all examined enzymes and transcription factors, compared with the non-treated control.

4. Discussion

Recently, anti-aging approaches have received enormous attention in the cosmeceuticals field due to aesthetic concerns. Skin aging is an unavoidable event in human life, however, extrinsic aging (photoaging) caused by environmental factors (such as sunlight, chemicals, hormones, lifestyle) accelerates premature skin aging[1-3]. To delay photoaging problems, many strategies for facilitating and maintaining a healthy and youthful skin have been investigated, including UV-protection, scavenging of free radicals, collagen-regeneration and pigmented-disturbance prevention[23,24]. Although anti-aging compounds have already been launched on markets, the side effects and the limitations of effectiveness are periodically noted[1]. To achieve a safe and highly effective anti-aging agent in cosmeceutical fulfillments, ingredients derived from natural sources are, thus, becoming greater targets of interest, compared to conventionally synthesized compounds[7-9].

RSC is a well-known marine organism that is rich in nutrition and health benefits. Consequently, RSC has been widely consumed as a traditional remedy and food supplement in Asian countries for many decades[14,17,18]. Moreover, many researchers have noted that RSC possesses important therapeutic activities such as anti-cancer, anti-inflammation, anti-oxidation, and anti-hyaluronidase[9,16-18]. However, there have not been any intensive investigations related to either anti-aging activity, or whether RSC-UE can function as a cosmetically active ingredient. Therefore, in the present study, RSC-UE from Jeju Island was investigated for its antioxidant and anti-melanogenic activities as we press toward finding natural bioactive ingredients in this novel cosmeceutical.

ESR is a technique based on a static magnetic field and microwaves detecting unpaired electrons including organic and inorganic free radicals. It is a convenient, fast, and efficient method which is able to identify tiny quantities of free radicals, providing greater accuracy compared to the conventional colorimetric assay[25,26]. ESR was used to measure the DPPH and alkyl free radical-scavenging activities of RSC-UE. DPPH, a free radical donor, is commonly used to evaluate the scavenging ability of antioxidants. The DPPH signal will be attenuated by coupling an unpaired electron which normally takes place at a nitrogen atom of the molecule[27,28]. Meanwhile, the alkyl free radical is primarily intermediated in many hydrocarbon reactions and is induced by AAPH[27,28]. Our study revealed that RSC-UE has a protective effect against harmful effects mediated by DPPH and alkyl free radicals, which confirms the findings of Husni *et al*. who reported that the heat reflux and pressurized solvent extraction of water and 70% ethanol of sea cucumber, *S. japonicus*, demonstrated antioxidant activities examined by the DPPH and 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activities[29].

In this investigation, cellular activity of RSC-UE was evaluated on human keratinocytes (HaCaT) and RSC-UE itself was not cytotoxic to HaCaT cell at any of the concentrations examined up to 400 μg/mL. AAPH is a peroxyl radical generator widely used to imitate the oxidative stress state[30]. After addition of AAPH to HaCaT, the elevation of intracellular ROS production and the oxidative stress induced by free radicals led to elevated rates of cell death[31]. Here, we used AAPH (10 mM) to determine whether RSC-UE exhibits any antioxidant effect on HaCaT cell. Given that in the presence of AAPH, half the cells died of oxidative stress. But our data indicating that RSC-UE can reduce free radical levels and protect HaCaT cells against cell death was still quite impressive.

Free radicals are unstable radicals containing an odd number of electrons which can initiate chain reactions[6,32]. ROS are active free radicals created during the interaction of oxygen with certain molecules, and these may occur in the form of singlet oxygen ($^1O_2$), superoxide anions (O^{2-}), hydroxyl anions ($OH^•$), or hydrogen peroxide (H_2O_2)[31,32]. Furthermore, ROS are ubiquitously found in various biological systems of aerobic organisms especially mitochondria[32,33]. However, excessive ROS production in the cell can harmfully attack many biochemical components including vital proteins, lipids and DNA thereby leading to compromised cell functions, cell senescence and ultimately, cell death[6]. In addition, the accumulation of ROS and oxidative stress is believed to be related to photoaging, inflammation, wound healing, tumorigenesis, and other unfavorable processes for skin health[29,31,33]. For example, Quan *et al*. discovered that oxidative stress through elevation of ROS can induce mitochondria DNA damage in dermal fibroblasts of aged human skin[34,35]. Hence, inhibition of ROS accumulation is able to interrupt degradation to the skin primary structural elements (collagen and elastin), and also prevent the creation of wrinkles and unhealthy skin appearance. Notably, one of the strategies for diminishing ROS is additional antioxidant consumption[4]. Under ideal circumstances, an antioxidant may provide protection against sun-induced damage, a delay in premature skin aging, reductions in skin inflammation and an improvement of skin appearance which are all anti-aging activities[4,13,23]. A dose-dependent decrease in ROS production and fluorescence intensity suggested that RSC-

UE prevents ROS generation or accumulation in AAPH-induced cells. Our findings showed that RSC-UE contributed to greater cell survival rate. Therefore, we conclude that RSC-UE with prominent antioxidant activity makes it a useful anti-aging compound for potential use in cosmeceuticals.

Melanin is a skin pigment capable of protecting the skin from UV damage, environmental pollutants and harmful chemicals. However, abnormal pigmentation, melanoma skin cancer as well as other unpleasant aesthetic changes are the results of overproduction of melanin in the skin[8,9]. Hydroquinone, a commercial whitening agent, has been used for hyperpigmentation disorder treatments and aesthetic purposes, however, various undesirable effects of hydroquinone were observed and presented as skin irritation, contact dermatitis, and exogenous ochronosis among dark-skinned people. A restriction of foods and drugs containing hydroquinone has therefore been enforced in USA since 2006[6,7,36]. Furthermore, the other natural whitening agents available in the market also have explicit limitations such as inadequate melanin inhibition (kojic acid), poor skin penetration during the clinical phase (arbutin) and a low stability during formulation development (glabridin)[8,9,36].

Melanogenesis consists of multiple steps that are catalyzed by tyrosinase, a crucial enzyme involved in melanin production. Tyrosinase plays a key role to catalyze melanin synthesis involving two consecutive reactions during melanogenesis; the first is hydroxylation of *L*-tyrosine to *L*-DOPA, and the second is oxidation of *L*-DOPA to DOPA-quinone, of which underreactions lead to skin pigmentation[37–39]. *In–vitro* tyrosinase assays in B16F10 cells were conducted to evaluate melanin inhibitory activity of RSC-UE. Our results suggested that RSC-UE preferentially interrupted melanogenesis. To further investigate the anti-melanogenesis effect of RSC-UE, α-MSH stimulated B16F10 cells treated with RSC-UE were examined in the present study.

α-MSH, an endogenous peptide hormone of the melanocortin family with an acetyltridecapeptide structure, is found in keratinocytes which stimulates melanogenesis in melanocytes[40,41]. In this study, RSC-UE attenuated cellular tyrosinase activities as well as melanin formation in α-MSH-induced melanoma cells. In agreement with our results, Husni *et al*. investigated the anti-melanogenesis effect of water (IC_{50}: 1.80–1.99 mg/mL) and 70% ethanol (IC_{50}: 0.49–0.61 mg/mL) extracts of *S. japonicus* by using *L*-3,4-dihydroxyphenylalanine and dopachrome isolated by HPLC as substrate. Their results indicated that ethyl-α-*D*-glucopyranoside and adenosine identified from RSC acted as key tyrosinase inhibitors against diphenolase activity on mushroom tyrosinase[42].

The *MITF* gene is a key regulator of melanogenesis activating the transcription of several melanogenic genes (tyrosinase, TRP-1, and TRP-2; namely dopachrome tautomerase). Consequently, α-MSH stimulates the melanogenesis signaling pathways by promoting the level of MITF which allows the upregulation of enzymatic gene expression of tyrosinase, TRP-1 and TRP-2, leading to enhanced skin pigmentation[39,41]. High relative amounts of MITF, tyrosinase, TRP-1 and TRP-2 were notably observed in cells treated with α-MSH, compared to the untreated cells. RSC-UE downregulated the transcription factor (MITF) and melanogenic proteins (tyrosinase, TRP-1, and TRP-2) in a dose-dependent manner, leading to reduced melanin production. In agreement with our results, Oh *et al*. has noted that a mixture of *S. japonicus* extract reduced the expression of MITF which also resulted in reduced protein expression of tyrosinase, TRP-1, and TRP-2 by inhibiting the phosphorylation of ERK and its expression pathway[43]. Recently, an ethyl acetate fraction of RSC with similar melanogenic inhibitory effects was also documented by Yoon *et al*[44]. Taken together, our results demonstrate effective melanogenic inhibition by RSC-UE, indicating a significant potential of RSC-UE for the development of a novel whitening agent candidate for use in the cosmeceutical arena.

In conclusion, RSC-UE demonstrates not only excellent antioxidant activity, but also a potential anti-melanogenic effect. Our study suggests that RSC-UE (*S. japonicus*) derived from Jeju Island may become an effective candidate for a novel anti-aging and whitening agent in cosmeceuticals.

Conflict of interest statement

We declare that there is no conflict of interest.

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Authors' contributions

SJH and SHL conceived and designed the experiments; YD, CJ, and EAK performed the experiments; CJ, JK and YD analyzed the data; SJH and JK contributed reagents/materials/analysis tools; CJ and SHL wrote the paper.

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