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Effect of Korean Red Sea Cucumber (*Stichopus japonicus*) on Melanogenic Protein Expression in Murine B16 Melanoma

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Abstract: In this study, we therefore assessed the effects of *S. japonicus* on melanogenic protein expression of murine B16 melanoma cells. We obtained an *S. japonicus* extract by treatment with 80% EtOH and then successively partitioned it with n-hexane, CH_2Cl_2, EtOAc, BuOH and water. Present results indicated that the EtOAc fraction of the *S. japonicus* extract (SJE) was effectively inhibited melanogenesis in murine B16 melanoma cells. We performed western blotting for melanogenic proteins to elucidate the mechanism of the effect of SJE on melanogenesis. SJE inhibited the expression of tyrosinase, Tyrosinase-Related Protein (TRP)-1 and TRP-2. We also confirmed that SJE decreased the protein level of melanocyte-specific isoform of Microphthalmia-Associated Transcriptional Factor (MITF), leading decreased expression of tyrosinase and tyrosinase-related genes in the B16 melanoma cells. To test the applicability of SJE to the human skin, we used MTT assay to assess the cytotoxic effects of SJE on human keratinocyte HaCaT cells. The SJE exhibited low cytotoxicity at 20 μg mL^{-1}. To test the applicability of *S. japonicus* as a cosmetic material with regard to its irritant or sensitization potential, we also performed human skin primary irritation tests, which showed no adverse reactions with *S. japonicus* usage. Hence, we suggest that *S. japonicus* be considered as a potential anti-melanogenic agent and may be effective for topical application for treating hyperpigmentation disorders.

Key words: Melanin, melanogenesis, MITF, *Stichopus japonicus*, tyrosinase, TRP-1, TRP-2

INTRODUCTION

Melanin, the major pigment of skin and hair colour in mammals, is synthesized by melanocytes within specialized organelles called melanosomes, which are then transferred from the melanocytes. It regulated by the melanogenic enzymes tyrosinase, Tyrosinase-Related Protein 1 (TRP-1) and Tyrosinase-Related Protein 2 (TRP-2). Tyrosinase is a copper-containing enzyme that converts L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and catalyzes the oxidation of DOPA into DOPA quinone. TRP-2, which functions as DOPA-chrome tautomerase, catalyzes the rearrangement of DOPA-chrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and TRP-1 oxidizes DHICA into a carboxylated indole-quinone. Tyrosinase is therefore the most important enzyme because melanin production is dependent on tyrosinase expression and activation (Zhu and Gao, 2008; Park *et al*., 2009; Yamaguchi and Hearing, 2009). Ultraviolet (UV) radiation, chronic inflammation and the release of abnormal α-melanocyte stimulating hormone (α-MSH) are well-known triggering factors for hyperpigmentation and inflammatory pigmentation (Im *et al*., 2002; Choi *et al*., 2005; Kim *et al*., 2008). Although, melanin plays an essential role in the protection of the skin against UV injury under normal physiological conditions, abnormal pigmentation, such as freckles, chloasma, age spots, melasma and sites of actinic damage, often causes serious skin problems (Jones *et al*., 2002; Kim *et al*., 2008). Therefore, the modulation of melanogenesis through medication and cosmetics is one of the important ways of treating abnormal skin pigmentation. A few known natural melanin synthesis inhibitors, including arbutin, polyphenol, stilbenes and kojic acid, have been the focus of research and their use as whitening agents is generally accepted. However, there is a clear need for the identification of safer and more efficient skin-whitening agents due to the carcinogenic potential and weak effect of the existing skin-whitening agents (Zhu and Gao, 2008; Chang, 2009; Kim *et al*., 2009a). Application of marine products in the cosmetics industry has continuously received a great deal

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of attention. In the course of *in vitro* screening of the extracts of various marine products for their action as melanin synthesis inhibitors, an ethanol extract of *Stichopus japonicus* was found to show anti-melanogenic activity. There are more than 100 species of sea cucumber, i.e., the phylum Echinodermata, that grow along the Korean and Japanese coasts and among them, over 20 species are edible (Saito *et al*., 2002). One such species found in Korea, *S. japonicus*, commonly known as marine ginseng (Haesam), is classified into 3 groups, namely blue, red and block sea cucumber. It has traditionally been used as a stamina food and possesses anti-tumour, immunoregulatory, anti-coagulant, anti-thrombin, wound healing and antiviral properties (Zhang, 1997; Fredalina *et al*., 1999; Lu and Wang, 2009). However, the effects of *S. japonicus* on melanogenesis have thus far not been examined. In this study, we therefore evaluated the inhibitory activity of *S. japonicus* on melanin biosynthesis and on melanogenic protein expression using B16 melanoma cells.

MATERIALS AND METHODS

Materials and solvent extraction: *S. japonicus* specimens were collected in September 2006 from Jeju Island, Korea. The specimen voucher (No. JBR-614) is deposited with the herbarium of Jeju Biodiversity Research Institute. For extraction, the material was first ground into a fine powder and freeze-dried using a vacuum freeze-dryer. The dried powder (50 g) was extracted with 80% ethanol (EtOH; 2 L) at room temperature for 24 h and then evaporated under vacuum. The evaporated EtOH extract (19.8 g) was suspended in water (1 L) and fractionated with 4 solvents: n-hexane (1 L), dichloromethane (CH_2Cl_2; 1 L), ethyl acetate (EtOAc; 1 L) and butanol (BuOH; 1 L). The yield and recovery of these 5 solvent fractions were as follows: n-hexane: 1.3 g and 6.8%, respectively; CH_2Cl_2: 1.1 g and 5.8%, respectively; EtOAc: 1.2 g and 6.3%, respectively; BuOH: 5.4 g and 28.4%, respectively; and H_2O: 8.9 g and 46.8%, respectively. The study was conducted on November 2008 to May 2009.

Cell culture: Cells of the murine melanoma cell line, B16F10 and the human keratinocyte cell line, HaCaT, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS; Gibco BRL) and 100 units mL^{-1} penicillin-streptomycin. The cells were incubated in the presence of 5% CO_2 at 37°C and were sub-cultured every 4 days.

Cell viability assay: Cell viability was measured by a conventional MTT assay. At 4 h prior to culture termination, 10 µL of MTT solution (10 mg mL^{-1} in phosphate-buffered saline, pH 7.4) was added and the cells were continuously cultured until termination of the experiment. Incubation was stopped by the addition of 15% Sodium Dodecyl Sulphate (SDS) into each well for solubilization of formazan and the Optical Density (OD) at 570 nm ($OD_{570\text{-}630}$) was measured with a microplate reader.

Melanin content assay: Cells of the murine melanoma cell line, B16F10, plated at 2.0×10^4 cells mL^{-1} were stimulated with α-MSH (100 nM) and then incubated with aliquots of the EtOH extract and each solvent fraction (25 µg mL^{-1}) at 37°C for 72 h; the cells were then washed in ice-cold phosphate-buffered saline. Briefly, the samples were incubated at 80°Cfor 1 h in 1 mL of 1 N NaOH/10% DMSO and then vortexed to solubilize the melanin; the absorbance at 450 nm was then measured. Further, the melanin content was determined based on the absorbance/µg of protein in the extract from each cell. The protein concentration of the cells was determined by using a protein assay kit (Pierce, Rockford, IL, USA).

Western blot analysis: B16F10 cells (1.0×10^5 cells mL^{-1}) were treated with α-MSH (100 nM) and the EtOAc fraction of *S. japonicus* (5, 10 and 20 µg mL^{-1}) for 24 h and the cellular proteins were extracted from the cells. Protein concentrations were determined by using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) with Bovine Serum Albumin (BSA) as a standard. The cell lysates (20-30 µg) were electrophoresed in SDS-polyacrylamide gels (8-12%) and the separated proteins were transferred on to PVDF membranes (Bio-Rad) for 2 h. The membranes were pre-incubated with the blocking solution (5% skimmed milk in Tris-buffered saline containing Tween-20) at room temperature for 2 h and then incubated with anti-rabbit tyrosinase, TRP-1, TRP-2 and MITF (1:1,000; Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5,000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 30 min. The bands were then visualized on an X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA). Human s kin primary irritation test: On the basis of certain inclusion and exclusion criteria, 32 healthy female Korean subjects (average age: 39.94±6.81 years; range: 20-49 years) were selected and written consent was obtained from each. None of the subjects had any history of allergic contact dermatitis and none had used any topical or systemic irritant preparation in the previous 1 month. An IQ Ultra Chamber® was secured to the back

of each subject with micropore tape. The round border of the chamber was placed firmly against the skin, causing tight occlusion of the test materials (Kim *et al*., 2009b; Yoon *et al*., 2009). The 80% EtOH extract of *S. japonicus* formulated with squalane was prepared as the negative control and applied at concentrations of 0.5 and 1%. The patches (chambers) were allowed to remain in place for 48 h. During this time, the subjects abstained from showering or performing any work or exercise that might have moistened or loosened the patches. The sites were read at 30 min and 24 h after removal of the patches; the readings were scored according to the Cosmetic, Toiletry and Fragrance Association (CTFA) guidelines.

Statistical analysis: Statistical differences were assessed by one-way analysis of variance (ANOVA) and Student's t-test. Data were expressed mean±SD of three independent experiments. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In melanocytes, melanin biosynthesis proceeds through a complex series of enzymatic and chemical reactions, involving 3 melanocyte-specific enzymes, namely TYR, TRP-1 and TRP-2. The promoter sequences of TYR, TRP-1 and TRP-2 share a highly conserved motif known as the M-box. MITF, a basic helix-loop-helix leucine zipper transcription factor, is believed to bind to the M-box, thereby transactivating TYR, TRP-1 and TRP-2 (Hearing and Jimenez, 1989; Hearing and Tsukamoto, 1991; Jung *et al*., 2009). Currently, numerous reported pharmacological and cosmeceutical agents inhibit tyrosinase or other melanogenic pathway targets (Choi *et al*., 2005; Parvez *et al*., 2007; Chang, 2009). However, because of the toxicity and low clinical activity of such agents, only a few are suitable for use as skin-whitening agents. The use of natural molecules derived from marine resources is on the rise due to their lack of side effects. Therefore, the potential of natural resources for cosmetic and medicinal purposes has been re-evaluated and investigated. During our search for effective melanin biosynthesis inhibitors in natural products, we found that *S. japonicus* has potent melanin biosynthesis inhibitory activity.

To investigate the influence of the *S. japonicus* fraction, the melanin content of the melanoma cells was measured after treatment of the cells with the *S. japonicus* fraction. As shown in Fig. 1a and b among the 5 fractions tested, the EtOAc fraction exhibited higher inhibitory activity towards melanin production than the $CHCl_3$, BuOH and H_2O fractions. The number of viable activated macrophages was not altered by the EtOAc fraction, as

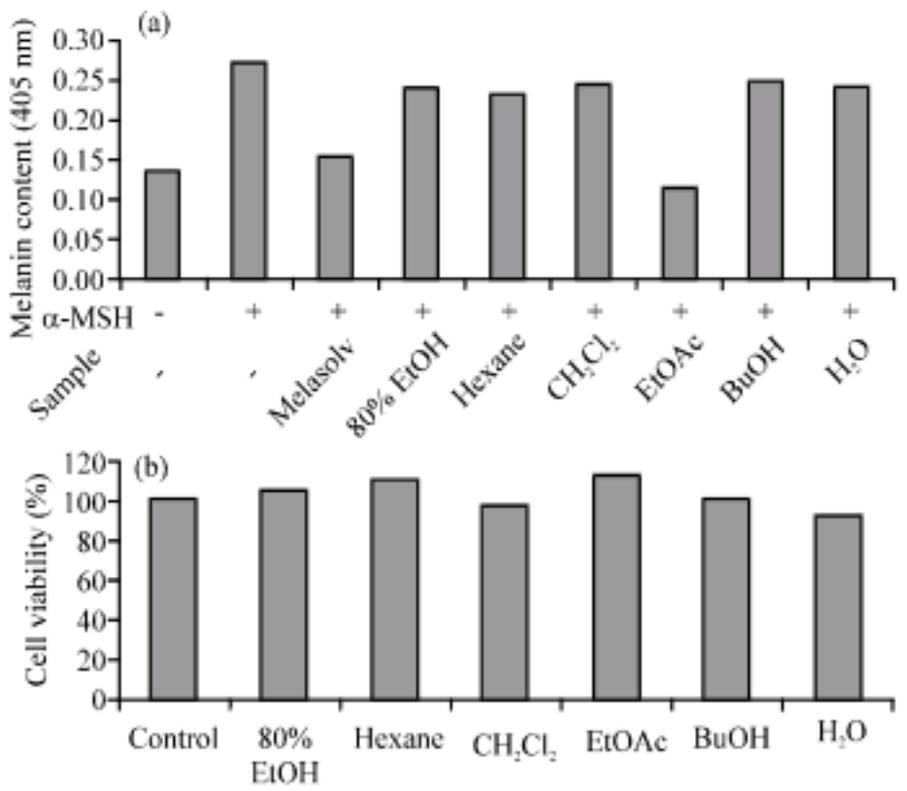

Fig. 1: (a, b) Effect of 80% EtOH and the solvent fractions of *S. japonicus* on melanogenesis and cell viability of the B16F10 cells. B16F10 cells (2.0×10^4 μg mL^{-1}) were pre-incubated for 18 h and the melanin content was assayed after incubation of the B16F10 cells treated with α-MSH (50 nM), melasolv (40 μM) and 80% EtOH and the solvent fractions of *S. japonicus* (20 μg mL^{-1}) for 72 h at 37°C in a 5% CO_2 atmosphere. The absorbance was measured at 405 nm by an ELISA. MTT assay was performed after incubation of the B16F10 cells treated with 80% EtOH and the solvent fractions of *S. japonicus* for 24 h at 37°C in a 5% CO_2 atmosphere. The absorbance was measured at 570 nm with a spectrophotometer (Power Wave; Bio-tek, Winooski, VT)

determined by the MTT assay, indicating that the inhibition of melanin production by the EtOAc fraction was not merely due to a cytotoxic effect. As a second step towards determining the effect of the *S. japonicus* EtOAc fraction (SJE) on melanogenesis, the B16 melanoma cells were subjected to a cell count and melanin content assay. The data from the MTT cell viability assay of the B16 cells are shown in Fig. 2a. At growth doses of 5, 10 and 20 μg mL^{-1} of SJE, the cell viability after 24 h treatment was 99.1, 98.3 and 93.5%, respectively. These data clearly indicate the non-cytotoxic nature of SJE. Thus, the inhibitory effect of SJE on the melanin content was assessed further. The commercial whitening agent, melasolv, was used as the positive control. The melanin content of the SJE-treated melanocytes is shown in Fig. 2b. As compared to the control group, 72-h treatment with SJE (5, 10 and 20 μg mL^{-1}) reduced the melanin content in a concentration-dependent manner.

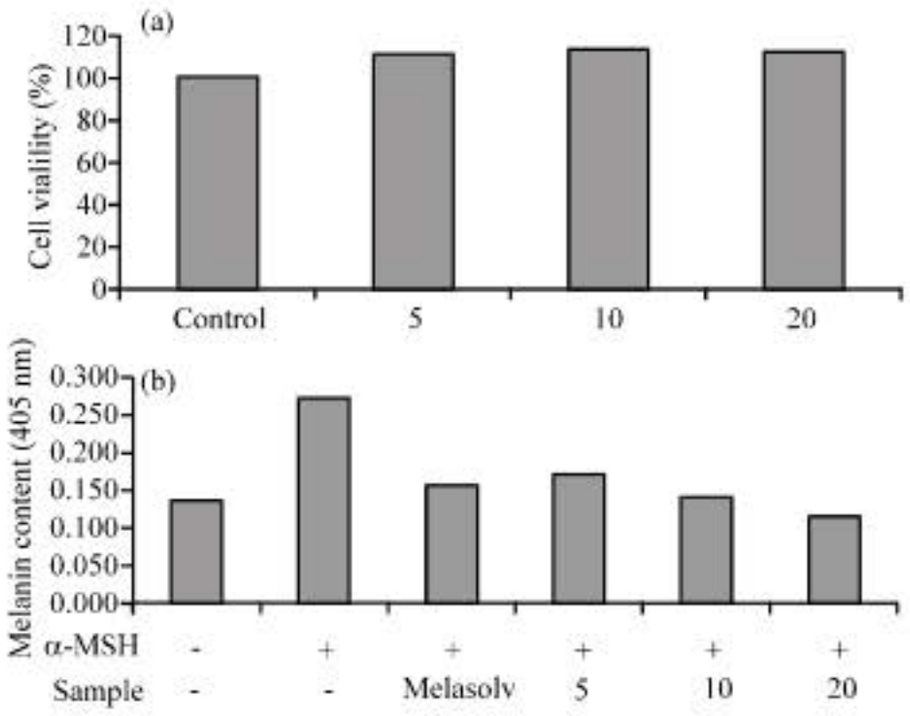

Fig. 2: Inhibitory effect of the *S. japonicus* EtOAc fraction on melanogenesis (a) and cell viability (b) of the B16F10 cells. B16F10 cells (2.0×10^4 μg mL^{-1}) were pre-incubated for 18 h and the melanin content was assayed after incubation of the B16F10 cells treated with α-MSH (50 nM), melasolv (40 μM) and the EtOAc fraction of *S. japonicus* (5, 10 and 20 μg mL^{-1}) for 72 h at 37°C in a 5% CO_2 atmosphere. The absorbance was measured at 405 nm by an ELISA. MTT assay was performed after incubation of the B16F10 cells treated with varying concentrations (5, 10 and 20 μg mL^{-1}) of the EtOAc fraction of *S. japonicus* for 24 h at 37°C in a 5% CO_2 atmosphere. The absorbance was measured at 570 nm with a spectrophotometer (Power Wave; Bio-tek, Winooski, VT)

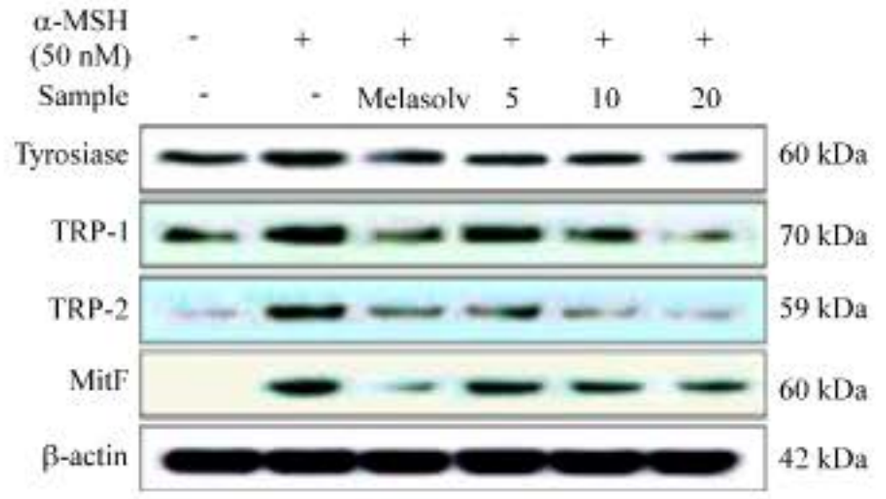

Fig. 3: Inhibitory effect of the *S. japonicus* extract on the protein level related to melanogenic factors in the B16F10 cells. B16F10 cells (1.0×10^5 cells mL^{-1}) were pre-incubated for 18 h and were stimulated with α-MSH (50 nM) in the presence of melasolv (40 μM) and the *S. japonicus* extract (5, 10 and 20 μg mL^{-1}) for 24 h. The protein level was determined by immunoblotting

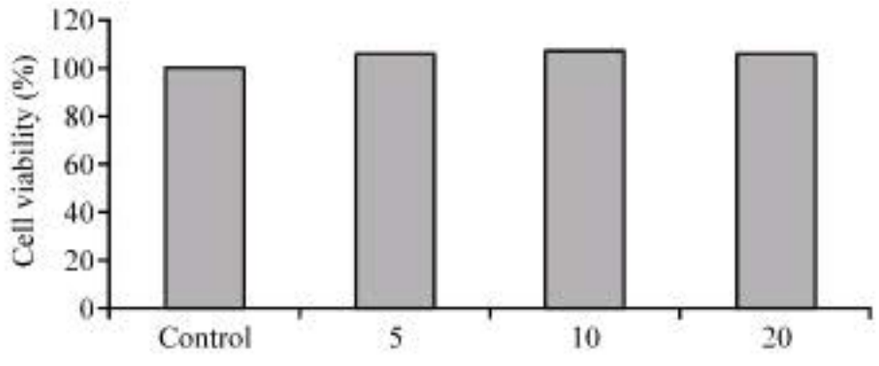

Fig. 4: Cell viability of the human keratinocyte HaCaT cells after treatment with the *S. japonicus* extract. MTT assay was performed after incubation of the B16F10 cells treated with varying concentrations (5, 10 and 20 μg mL^{-1}) of the *S. japonicus* extract for 24 h at 37°C in a 5% CO_2 atmosphere. The absorbance was measured at 570 nm with a spectrophotometer (Power Wave; Bio-tek, Winooski, VT)

To study the hypopigmentary effect of SJE, we evaluated the mechanism of action of SJE with respect to melanogenesis, since melanin is a heteropolymer produced within melanosomes by the TYR enzyme, which acts on the TYR precursor material found in melanocytes themselves. It has been reported that other factors, such as metal ions and TRP enzymes (TRP-1 and TRP-2), also affect the production of melanin. These proteins constitute a specific family of membrane proteins that are structurally related, but have distinct enzymatic functions (Negroiu *et al*., 2000; Huang *et al*., 2008). Therefore, we performed western blot analysis after 24 h SJE treatment to evaluate the effect of SJE on these proteins. Exposure of melanocytes to varying concentrations of SJE (5, 10 and 20 μg mL^{-1}) resulted in dose-dependent decreases in TYR, TRP-1 and TRP-2 expression (Fig. 3). The positive control, melasolv, also reduced TYR, TRP-1 and TRP-2 protein levels. Taken together, these observations suggest that SJE reduced the expression of the 3 TRPs in a concentration-dependent manner. Based on the present study, SJE was found to decrease the levels of the pigment-related proteins, TYR, TRP-1 and TRP-2. MITF effectively transactivates the TYR, TRP-1 and TRP-2 genes and is considered to be a key regulator of melanocyte development. Therefore, the effect of SJE on MITF expression was also evaluated in this study. As shown in Fig. 3, based on the western blot analysis, the upstream transcription factor MITF was down-regulated by SJE in a dose-dependent manner.

We also examined the cytotoxic effect of SJE on human keratinocyte HaCaT cells to evaluate whether it would have a cytotoxic effect on human skin cells when applied as a therapeutic agent. In such a case, SJE would not be suitable for use as a therapeutic agent. As shown in Fig. 4, the cell viability was almost 100% on exposure to

Table 1: Results of the human skin primary irritation test (n = 32)

Test materials	No. of responders	48 h					72 h				Reaction grade[a]		
		1+	2+	3+	4+	±	1+	2+	3+	4+	48 h	72 h	Mean
Squalane	0	[b]-	-	-	-	-	-	-	-	-	0.0	0.0	0.0
S. japonicus extract (0.5%)	0	-	-	-	-	-	-	-	-	-	0.0	0.0	0.0
S. japonicus extract (1%)	0	-	-	-	-	-	-	-	-	-	0.0	0.0	0.0

[a]Reaction grade = Σ[{Grade×No. of responders}/4 (maximum grade)×32 (total subjects)}]×100× (1/2). [b]No reaction.

SJE at concentrations below 20 μg mL^{-1}. These data suggest that SJE has low cytotoxicity against mammalian cell lines. From the point of view of the application of the *S. japonicus* extract to human skin as a topical agent, we evaluated the irritant effect of the extract on human skin by conducting patch tests as a part of this study. As shown in Table 1, none of the 32 subjects experienced any allergic reaction, based on the 48 and 72 h readings. Moreover, we did not observe any severe adverse reactions such as erythema, burning, or pruritus related to the topical treatment with the *S. japonicus* extract. In conclusion, present results demonstrate that *S. japonicus* extract can reduce melanin production and is safe for therapeutic use.

CONCLUSION

In the present study, the EtOAc fraction of *Stichopus japonicus* showed potent cellular anti-melanogenic activity on B16 melanoma cells. Our results indicate that SJE reduces the activity of TYR, TRP-1 and TRP-2, particularly that of TRP-1 and TRP-2, at the protein level in melanocytes. Melanogenesis is regulated by a series of enzymes that are under the control of MITF. Further, SJE also reduces the MITF protein level. In order to test the applicability of *S. japonicus* as a cosmetic material with regard to its irritant or sensitization potential, human skin primary irritation tests were performed on 32 volunteers; no adverse reactions were observed with the use of *S. japonicus*. Thus, *S. japonicus* may prove to be an effective skin-whitening agent for use in skin care cosmetics or as a hypopigmentation agent.

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REFERENCES

Chang, T.S., 2009. An updated review of tyrosinase inhibitors. Int. J. Mol. Sci., 10: 2440-2475.

Choi, H., S. Ahn, B.G. Lee, I. Chang and J.S. Hwang, 2005. Inhibition of skin pigmentation by an extract of *Lepidium apetalum* and its possible implication in IL-6 mediated signaling. Pigment Cell Res., 18: 439-446.

Fredalina, B.D., B.H. Ridzwan, A.A. Zainal Abidin, M.A. Kaswandi and H. Zaiton *et al*., 1999. Fatty acid compositions in local sea cucumber, *Stichopus chloronotus*, for wound healing. Gen. Pharmacol., 33: 337-340.

Hearing, V.J. and M. Jiménez, 1989. Analysis of mammalian pigmentation at the molecular level. Pigment Cell Res., 2: 75-85.

Hearing, V.J. and K. Tsukamoto, 1991. Enzymatic control of pigmentation in mammals. FASEB J., 5: 2902-2909.

Huang, Y.H., T.H. Lee, K.J. Chan, F.L. Hsu, Y.C. Wu and M.H. Lee, 2008. Anemonin is a natural bioactive compound that can regulate tyrosinase-related proteins and mRNA in human melanocytes. J. Dermatol. Sci., 49: 115-123.

Im, S., J. Kim, W.Y. On and W.H. Kang, 2002. Increased expression of alpha-melanocyte-stimulating hormone in the lesional skin of melasma. Br. J. Dermatol., 146: 165-167.

Jones, K., J. Hughes, M. Hong, Q. Jia and S. Orndorff, 2002. Modulation of melanogenesis by aloesin: A competitive inhibitor of tyrosinase. Pigment Cell Res., 15: 335-340.

Jung, E., J. Lee, S. Huh, J. Lee, Y.S. Kim, G. Kim and D. Park, 2009. Phloridzin-induced melanogenesis is mediated by the cAMP signaling pathway. Food Chem. Toxicol., 47: 2436-2440.

Kim, Y.J., K.S. Kang and T. Yokozawa, 2008. The anti-melanogenic effect of pycnogenol by its anti-oxidative actions. Food Chem. Toxicol., 46: 2466-2471.

Kim, J.H., M.R. Kim, E.S. Lee and C.H. Lee, 2009a. Inhibitory effects of calycosin isolated from the root of *Astragalus membranaceus* on melanin biosynthesis. Biol. Pharm. Bull., 32: 264-268.

Kim, J.Y., D.S. Kim, E.J. Yang, W.J. Yoon and J.S. Baik *et al*., 2009b. Green alga *Ulva pertusa* inhibits nitric oxide and prostaglandin-E_2 formation in murine macrophage RAW 264.7 cells. J. Appl. Biol. Chem., 52: 38-40.

Lu, Y. and B.L. Wang, 2009. The research progress of antitumorous effectiveness of *Stichopus japonicus* acid mucopolysaccharide in north of China. Am. J. Med. Sci., 337: 195-198.

Negroiu, G., R.A. Dwek and S.M. Petrescu, 2000. Folding and maturation of tyrosinase-related protein-1 are regulated by the post-translational formation of disulfide bonds and by N-glycan processing. J. Biol. Chem., 275: 32200-32207.

Park, H.Y., M. Kosmadaki, M. Yaar and B.A. Gilchrest, 2009. Cellular mechanisms regulating human melanogenesis. Cell Mol. Life Sci., 66: 1493-1506.

Parvez, S., M. Kang, H.S. Chung and H. Bae, 2007. Naturally occurring tyrosinase inhibitors: Mechanism and applications in skin health, cosmetics and agriculture industries. Phytother. Res., 21: 805-816.

Saito, M., N. Kunisaki, N. Urano and S. Kimura, 2002. Collagen as the major edible component of sea cucumber (*Stichopus japonicus*). J. Food Sci., 67: 1319-1322.

Yamaguchi, Y. and V.J. Hearing, 2009. Physiological factors that regulate skin pigmentation. Biofactors, 35: 193-199.

Yoon, W.J., Y.M. Ham, B.S. Yoo, J.Y. Moon, J. Koh and C.G. Hyun, 2009. *Oenothera laciniata* inhibits lipopolysaccharide induced production of nitric oxide prostaglandin E_2 and proinflammatory cytokines in RAW264.7 macrophages. J. Biosci. Bioeng., 107: 429-438.

Zhang, G., 1997. The antithrombin action of *Stichopus japonicus* acid mucopolysaccharide (Sjamp) is mediated by heparin cofactor II. Zhonghua Xue Ye Xue Za Zhi., 18: 126-129.

Zhu, W. and J. Gao, 2008. The use of botanical extracts as topical skin-lightening agents for the improvement of skin pigmentation disorders. J. Investig. Dermatol. Symp. Proc., 13: 20-24.