THE JOURNAL OF DERMATOLOGY

doi: 10.1111/j.1346-8138.2010.00983.x

Journal of Dermatology 2011; 38: 354–363

ORIGINAL ARTICLE

Screening of marine algae for potential tyrosinase inhibitor: Those inhibitors reduced tyrosinase activity and melanin synthesis in zebrafish

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ABSTRACT

In order to find new anti-browning and whitening agents in this study, we investigated 43 indigenous marine algae for tyrosinase inhibitory activity. The extracts from *Endarachne binghamiae*, *Schizymenia dubyi*, *Ecklonia cava* (EC) and *Sargassum silquastrum* (SS) evidenced potent tyrosinase inhibitory activity similar to that of positive control, kojic acid. Among those marine algae, EC and SS are distributed abundantly on Jeju Island. Therefore, we selected those two species for further studies. Our results evidenced that both species reduced cellular melanin synthesis and tyrosinase activity. On the other hand, we utilized zebrafish as an alternative *in vivo* model. All the tested samples evidenced excellent inhibitory effects on the pigmentation of zebrafish, most likely due to their potential tyrosinase inhibitory activity. In simultaneous *in vivo* toxicity tests, no toxicity was observed in either algal species, on the other hand, toxicity was observed in positive controls. These results provided that EC and SS extract could be used as an ingredient for whiting cosmetics and that zebrafish is an alternative *in vivo* model.

Key words: Korea, marine algae, melanin synthesis inhibitor, tyrosinase inhibitor, zebrafish.

INTRODUCTION

Tyrosinase inhibitors may be clinically useful for the treatment of some dermatological diseases associated with melanin hyperpigmentation and important in cosmetics for depigmentation.[1,2]

Several chemical compounds of plant origin have been reported as tyrosinase inhibitors. Ellagic acid,[3] oxyresveratrol,[4] chlorophorin and norartocarpanone[5] were described for their tyrosinase inhibition properties. Despite a large number of tyrosinase inhibitors reported, the identification and isolation of tyrosinase inhibitors from natural sources are currently one of the most important approaches.[6] Furthermore, it has been reported that tyrosinase might contribute to the dopamine neurotoxicity and neurodegeneration associated with Parkinson's disease.[7] These facts led us to focus our research work on the exploration of natural tyrosinase inhibitors from marine algae.

In recent years, many marine resources have attracted attention in the search for bioactive compounds for the development of new drugs and health foods. Marine algae are known to be rich in vitamins, minerals and a variety of functional polysaccharides and polyphenols.[8–12]

The vertebrate zebrafish (*Danio rerio*) is a small tropical freshwater fish that has emerged as a highly advantageous vertebrate model organism because of its small size, large clutches, transparency, low cost and physiological similarity to mammals.[13,14] Traditionally, zebrafish has been used in the fields of molecular genetics and developmental biology.[15,16] However, its value as a model organism for drug discovery and toxicological studies has been recognized

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Received 21 June 2009; accepted 11 May 2010.

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recently.[17,18] The application of drugs and/or small molecules to zebrafish is simple because the early stage embryo rapidly absorbs small molecular compounds diluted in the bathing media through the skin and gills. In contrast, relatively late stage zebrafish (from 7 days post-fertilization [dpf] to the adult stage) absorb the compounds orally rather than percutaneously.[19] Therefore, the use of early stage larva provides another advantage of testing percutaneous effects of medicinal and/or cosmetic compounds. In addition, zebrafish has melanin pigments on the surface, allowing simple observation of the pigmentation process without complicated experimental procedures.[20]

The aim of this study is to develop natural whitening materials from the marine algae in Jeju, Korea against tyrosinase and melanin synthesis activity, and also to demonstrate the zebrafish as an alternative *in vivo* model.

METHOD

Algal materials

Algae were collected from February to April 2008 along the coast of Jeju Island, Korea. The algae were identified at the Faculty of Applied Marine Science in Jeju National University. Salt, epiphytes and sand were removed using freshwater. Finally, the algae were carefully rinsed with freshwater and stored in a refrigerator at −20°C. The frozen samples were lyophilized and homogenized with a grinder before extraction.

Extract preparation

Aqueous extracts from the algae (1 g dry weight) were prepared with 100 mL of distilled water for 24 h under continuous shaking at room (20°C) and high (70°C) temperature, and then the extracts were concentrated under a vacuum in a rotary evaporator at 40°C.

Screening of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined based on the previously described procedure[21] with a slight modification. In brief, the test reaction mixture comprised each algae extract, mushroom tyrosinase (200 units; Sigma-Aldrich, St Louis, MO, USA) and L-tyrosine (1.5 mmol/L) in 0.1 mol/L potassium phosphate buffer (pH 6.8). The reaction mixture was incubated at 37°C for 12 min, and the absorption was measured at 475 nm. The absorbance of the same mixture without tyrosinase was used as the control. Kojic acid and arbutin were used as positive controls. The optical density of the inhibition in the control was considered to represent 100%. The data are expressed as mean percentages and the results were repeated in triplicate.

IN VITRO CELL-BASED ASSAY

Cell culture

The murine B16/F10 melanocyte was cultured in Dulbecco's modified Eagle's medium (Gibco BRL Co., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and were maintained in a humidified incubator with 5% CO_2.

Cytotoxicity assay

Cell viability was then estimated through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells.[22] The cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/mL. After 16 h, the cells were treated with melanogenic inhibitors. Thereafter, the medium was removed and the cells were incubated with 0.5 mg/mL of MTT solution. After incubation for 3 h at 37°C and 5% CO_2, the supernatant was removed and formation of formazan was measured at 540 nm with a microplate reader (Packard Spectrocount, Meriden, CT, Austria).

Measurement of melanin content in melanocyte

Melanin content was measured as reported by Son *et al.*[23] with slight modifications. The cells were treated with the extracts for 24 h. After the treatment, the cells were detached by incubation with trypsin/ethylenediamine tetraacetic acid. After precipitation, the color of the cell pellets was evaluated visually, and cell pellets containing a known number of cells

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were solubilized in 1 mol/L NaOH at 100°C for 30 min. Spectrophotometric analysis of melanin content was performed at 490-nm absorbance. The optical density of the inhibition in the control was considered to represent 100%. The data are expressed as mean percentages and the results were repeated in triplicate.

Tyrosinase activity of marine algae in melanocyte

Tyrosinase inhibitory activity in B16 cells was determined based on the previously described procedure[21] with a slight modification. In brief, cells were plated in 24-well plates at a density of 5×10^4 cells/mL. B16 cells were incubated in the presence or absence of melanogenic inhibitors for 24 h. The cells were lysed in Pro-Prep protein extraction solution (Intron, Seoul, Korea). Cellular extracts were clarified by centrifugation at 12 000 *g* for 30 min at 4°C. The supernatant (80 μL) and 20 μL of L-tyrosine (1.5 mmol/L) were placed in a 96-well plate, and the reaction mixture was incubated at 37°C for 12 min, and the absorbance was measured at 475 nm. The absorbance of the same mixture without tyrosinase was used as the control. Kojic acid and arbutin were used as positive controls. The optical density of the control was considered to represent 100%. The data are expressed as mean percentages and the results were conducted in triplicate.

IN VIVO ASSAY

Origin and maintenance of parental zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Seoul, Korea) and 10 fish were kept in a 3-L acrylic tank at 28.5°C, with a 14:10 h light : dark cycle. Zebrafish were fed three times a day, 6 day/week, with Tetramin flake food (SEWHAPET Food Co., Seoul, Korea) supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 min.

Zebrafish pigmentation evaluating

Synchronized embryos were collected and arrayed by pipette, 10–15 embryos/well, in 24-well plates containing 475 μL embryo medium. Test compounds were dissolved in 1% dimethylsulfoxide and then added to the embryo medium from 9–35 h post-fertilization (hpf). The effects on the pigmentation of zebrafish were observed under the microscope. In all experiments, 0.2 mmol/L 1-phenyl-2-thiourea (PTU) and 20 mmol/L arbutin were used to generate transparent zebrafish without interfering in the developmental process,[24] and considered as a standard positive control. Phenotype-based evaluations of body pigmentation were carried out at 35 dpf. For observation, embryos were dechorionated in 2 mg/mL pronase (non-specific enzyme; Sigma, St Louis, MO, USA), anesthetized in tricaine methanesulfonate solution (Sigma), and photographed under a SZX9 microscope (Olympus, Tokyo, Japan).

Tyrosinase activity of marine algae in zebrafish embryos

Tyrosinase inhibitory activity was spectrometrically determined as described previously.[20] Briefly, approximately 100 zebrafish embryos were treated without or with melanogenic inhibitors (*Ecklonia cava* [EC], *Sargassum silquastrum* [SS], PTU or arbutin) from 9–35 dpf, and sonicated in Pro-Prep protein extraction solution (Intron). The lysate was clarified by centrifuging at 10 000 *g* for 5 min. A 250 μg of total protein in 100 μL of lysis buffer was transferred into the 96-well plate, and 100 μL of 1.5 mmol/L L-tyrosine was added. The control well contained 100 μL of lysis buffer and 100 μL of 1.5 mmol/L L-tyrosine. After incubation for 60 min at 28°C, absorbance was measured at 475 nm using the micro-reader (Packard Spectrocount). PTU and arbutin were used as positive controls. The optical density of the control was considered to represent 100%. The data are expressed as mean percentages and the results were repeated for three times in triplicate.

Melanin contents of zebrafish embryos

For the determination of melanin content, briefly, about 100 zebrafish embryos were treated without or with melanogenic inhibitors (ES, SS, PTU or arbutin) from 9 to 35 dpf, and sonicated in Pro-Prep protein extraction solution (Intron). After the centrifugation, pellet was dissolved in 1 mL of 1 N NaOH at 100°C for 30 min. The mixture was then vigorously vortexed to solubilize the melanin pigment. Optical density of the supernatant was measured at 490 nm, and the

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result was compared with the control which was considered to represent 100%. The melanin content was calibrated by protein amount, and the results were repeated for three times in triplicate.

Measurement of heartbeat rate

The heartbeat rate of both atrium and ventricle was measured at 35 dpf to determine the sample toxicity.[20] Counting and recording of atrial and ventricular contraction were performed for 3 min under the microscope, and results were presented as the average b.p.m. Arbutin (20 mmol/L) and PTU (0.2 mmol/L) were used as the positive controls. Concentration of the tested sample was 100 μg/mL.

Statistical analysis

The data are expressed as the mean ± standard error and one-way ANOVA (using SPSS ver. 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by the Student's *t*-test ($P < 0.05$, $P < 0.01$).

RESULTS

Screening of tyrosinase inhibitory activity of marine algae extracts

A total of samples from 43 marine algae, prepared as aqueous extracts at 20°C and 70°C, have been screened for their potential tyrosinase inhibitory activities. Table 1 summarizes the results of the tyrosinase inhibition of several tested marine algae extracts. Out of the tested extracts, *Endarachne binghamiae* (EB), *Schizymenia dubyi* (SD), *Ecklonia cava* (EC) and SS showed relatively higher inhibitory activity than those of the other extract, therefore, it was used for further analysis. The half maximal inhibitory concentration (IC_{50}) values of those algae are shown in Table 2. The extracts at 20°C from SD evidenced the most profound tyrosinase inhibitory activity, with an IC_{50} value of 9.08 μg/mL. The other algae extracts, including the extracts at 70°C from EC, EB and the extract at 20°C from SS evidenced relatively strong tyrosinase inhibitory activities with IC_{50} values of 18.00, 27.16 and 19.85 μg/mL, respectively (Table 2), whereas arbutin and kojic acid inhibited tyrosinase activity, with IC_{50} values of 13.86 and 1.21 μg/mL, respectively. Those marine algae evidenced anti-tyrosinase activity similar to that of arbutin. Interestingly, the tyrosinase inhibitory effect of SD extract was more profound than that of arbutin. Among the marine

Table 1. Tyrosinase inhibitory activity (%) of marine algae extract

	Inhibitory activity (%)	
Scientific name	20°C	70°C
Rhodophyta		
Gracilaria verrucosa	28.18 ± 1.12	30.55 ± 0.99
Grateloupia elliptica	25.30 ± 1.03	29.82 ± 0.98
Grateloupia lanceolate	23.55 ± 1.33	25.72 ± 0.90
Sinkoraena lancifolia	27.30 ± 0.33	30.00 ± 0.98
Grateloupia filicina	25.96 ± 1.00	27.88 ± 1.11
Capopeltis affinis	27.87 ± 0.02	32.48 ± 1.00
Laurencia okamurae	27.05 ± 1.77	25.00 ± 1.00
Chondria cassicaulis	27.62 ± 1.09	40.94 ± 1.22
Ahnfeltiopsis flabelliformis	17.82 ± 0.50	31.06 ± 1.12
Lomentaria catenata	21.03 ± 0.76	32.20 ± 2.00
Pterocladiella capillacea	30.49 ± 0.99	41.02 ± 1.08
Prionitis cornea	10.27 ± 0.85	42.31 ± 1.23
Gloiopeltis furcata	9.95 ± 1.00	28.08 ± 1.11
Chondrophycus undulatus	36.30 ± 0.89	24.87 ± 1.57
Schizymenia dubyi	90.75 ± 1.04	30.29 ± 0.98
Gelidium amansii	73.87 ± 1.02	17.14 ± 0.54
Lithophyllum okamurai	12.47 ± 0.05	33.98 ± 1.11
Chondrus crispus	12.81 ± 0.17	31.14 ± 1.07
Acrosorium flabellatum	12.00 ± 0.56	32.83 ± 1.01
Polysiphonia japonica	26.25 ± 0.98	13.74 ± 0.76
Chlorophyta		
Codium contractum	1.64 ± 0.05	18.61 ± 1.00
Ulva conglobata	5.54 ± 0.22	3.52 ± 0.79
Ulva pertusa	4.15 ± 0.50	14.78 ± 1.00
Enteromorpha compressa	0.26 ± 0.01	25.24 ± 1.11
Monostroma nitidum	46.10 ± 1.00	7.39 ± 1.12
Phaeophyta		
Dictyota dichotoma	50.93 ± 1.21	25.01 ± 1.01
Endarachne binghamiae	22.80 ± 1.00	81.26 ± 1.01
Undaria pinnatifida	24.09 ± 1.11	23.01 ± 1.01
Hizikia fusiforme	15.65 ± 0.99	12.67 ± 1.00
Laminaria ochotensis	25.49 ± 0.93	21.76 ± 1.00
Sargassum thunbergii	23.96 ± 0.92	23.14 ± 1.02
Colpomenia sinuosa	30.82 ± 0.90	25.91 ± 1.03
Petrospongium rugosum	48.30 ± 1.00	61.10 ± 1.00
Sargassum horneri	24.28 ± 1.01	11.45 ± 0.98
Sargassum coreanum	30.52 ± 1.01	31.26 ± 0.99
Sargassum siliquastrum	70.75 ± 1.00	43.60 ± 0.97
Myagropsis myagroides	49.77 ± 0.99	34.68 ± 0.80
Padina arborescens	17.10 ± 0.97	21.08 ± 0.99
Sagassum piluliferum	11.89 ± 0.98	50.81 ± 1.11
Pachydictyon sp.	27.38 ± 0.88	37.67 ± 0.99
Scytosiphon lomentaria	15.05 ± 1.00	24.07 ± 1.00
Desmarestia tabacoides	12.93 ± 1.12	28.15 ± 1.33
Ecklonia cava	12.63 ± 0.80	73.22 ± 0.80
Controls		
Kojic acid	97.61 ± 2.00	
Arbutin	96.95 ± 1.50	

Data are presented as average value of three different experiment and expressed as mean ± standard error. Sample concentrations were 100 μg/mL.

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Table 2. Half maximal inhibitory concentration (IC_{50}) values of tyrosinase inhibitory activity of the melanogenic inhibitors

Items	IC_{50} (μg/mL)
EB	27.16
SD	9.08
EC	18.00
SS	19.85
Arbutin	13.86
Kojic acid	1.21

EB, *Endarachne binghamiae*; SD, *Schizymenia dubyi*; EC, *Ecklonia cava*; SS, *Sargassum silquastrum*.

algae tested herein, EC and SS are distributed abundantly on Jeju Island in Korea. Therefore, we applied both extracts in *in vitro* cell and *in vivo* experiments.

IN VITRO CELL-BASED ASSAY

Effects of melanogenic inhibitors on cell viability

To exclude the possibility that inhibitory effects of EC, SS and arbutin on melanogenesis might be caused by the inhibition of cell growth, we compared the number of cells grown in the presence and absence of EC, SS and arbutin. EC, SS and arbutin did not seem to inhibit cell viability at a concentration below 100 μg/mL. However, we observed the cell viability with MTT assay after EC, SS and arbutin treatment with different concentrations. After treatment of both algae (EC and SS), a 50% of cell viability presenting concentration were 387 and 193 μg/mL, respectively, while a 50% of cell viability showing concentration of arbutin was more than 680.75 μg/mL. The survival rate of the cell was 88.43% at this concentration (680.75 μg/mL of arbutin). These results indicate that EC, SS and arbutin had no cytotoxicity at any concentration used in this study.

Effects of melanogenic inhibitors on tyrosinase activity and melanin synthesis in melanocyte

In order to evaluate the effects of algae, including EC and SS, on melanin synthesis and cellular tyrosinase inhibitory activity, we measured the melanin contents of melanocytes cultured in the presence of the test substances at 100 μg/mL concentrations. In Figure 1, the pigmentation of melanocytes was inhibited significantly ($P < 0.05$) by marine algae; arbutin and kojic acid were employed as the positive controls, and as

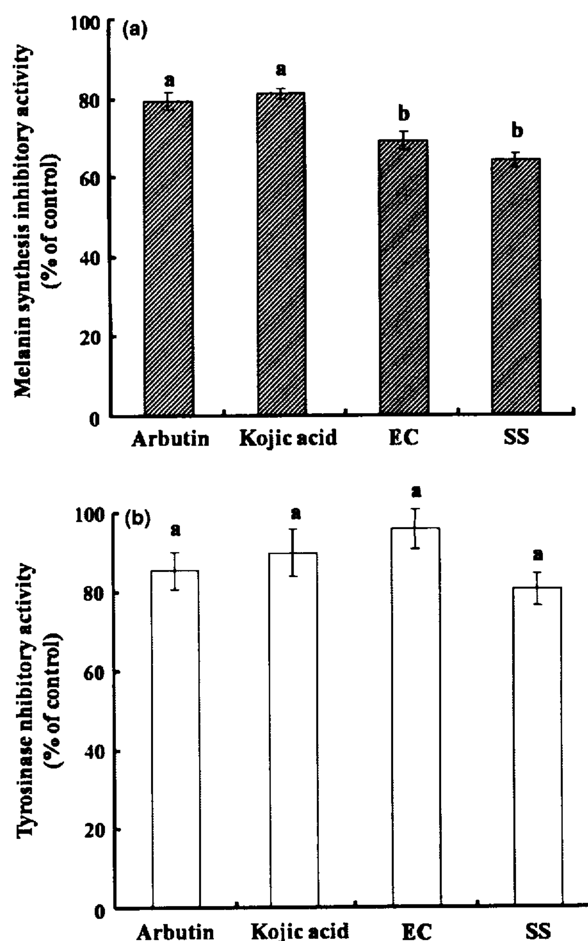

Figure 1. Effects of melanogenic inhibitors on melanin synthesis and tyrosinase activity in melanocytes. After treatment with each melanogenic inhibitor for 24 h, melanin contents were measured (a) and tyrosinase inhibitory activities were determined (b). EC is *Ecklonia cava* and SS is *Sargassum siliquastrum*. Arbutin and kojic acid were utilized as positive controls. Concentration of the tested sample was 100 μg/mL. The experiments were conducted in triplicate and the data are expressed as mean ± standard error. Statistical evaluation was performed to compare the experimental groups and control groups. [a,b]Values having different superscripts are significantly different at $P < 0.05$.

anticipated they inhibited melanin synthesis. Although the inhibition rate of melanin synthesis by algae is relatively lower than that observed in the positive controls, their tyrosinase inhibitory activities were similar to those of the positive controls. Additionally, EC exhibited more profound tyrosinase inhibitory activity (92%) than was observed with the positive controls (arbutin and kojic acid inhibited 80% and 81.5%,

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Table 3. Half maximal inhibitory concentration (IC_{50}) values of melanin synthesis inhibitory activity on B-16 cell line

Items	IC_{50} (μg/mL)
EC	58.65
SS	72.68
Arbutin	37.37
Kojic acid	36.65

EC, *Ecklonia cava*; SS, *Sargassum silquastrum*.

respectively). As shown in Table 3, the IC_{50} values of melanin synthesis inhibitory activity for EC and SS on the B16 cell line were 58.65 and 72.68 μg/mL, respectively, whereas the IC_{50} values of melanin synthesis inhibitory activity for arbutin and kojic acid were 37.37 and 36. 65 μg/mL, respectively.

IN VIVO ASSAY

Effects of melanogenic inhibitors on tyrosinase activity and melanin synthesis in zebrafish embryo

In order to estimate the inhibitory activities, we measured the tyrosinase activity and total melanin content using whole zebrafish extracts. We noted substantial reductions in tyrosinase activity and total melanin contents after the treatment with extracts from marine algae (Figs 2,3). PTU as a positive control, as anticipated, reduced both tyrosinase activity (69%) and total melanin contents (72%) to a marked degree. Arbutin also reduced tyrosinase activity (57%) and total melanin contents (61%). On the other hand, EC and SS also inhibited both tyrosinase activity (48% and 50%, respectively) and total melanin contents (43% and 50%, respectively), (Fig. 2). This is showed in Figure 3, which contains an image of the morphological findings. The positive controls, including PTU and arbutin, evidenced a remarkable inhibition of trunk and yolk sac pigmentation. When EC and SS were used, the melanin shrank on the surface of the trunk in a dose-dependent manner. In particular, the yolk sac pigmentation was inhibited dramatically after treatment with the algae extracts.

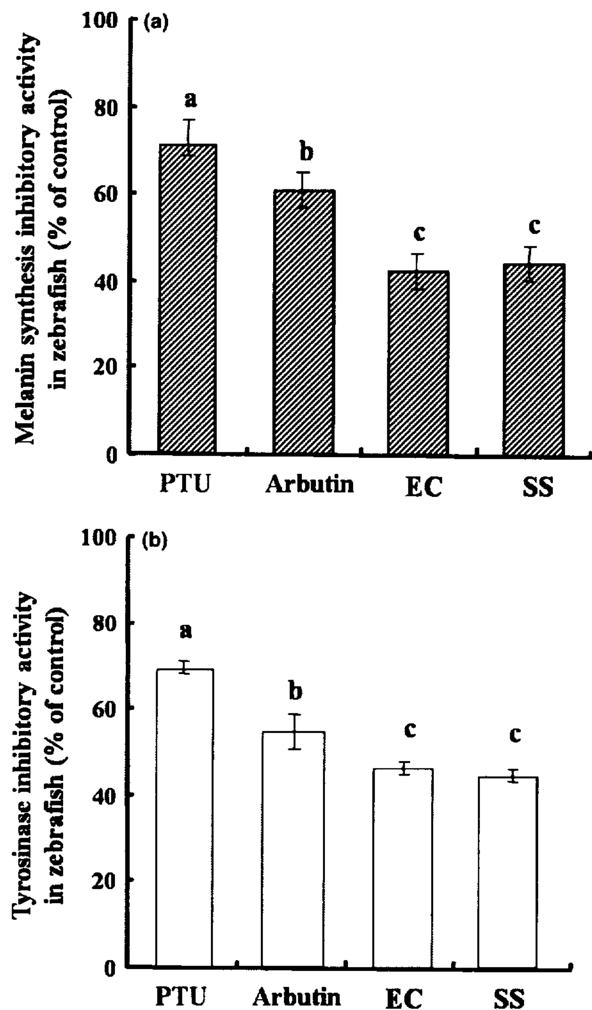

Figure 2. Effects of melanogenic inhibitors on melanin synthesis and tyrosinase activity in zebrafish embryos. Zebrafish embryos were treated with each melanogenic inhibitor from 9–35 days post-fertilization (dpf) and then melanin contents were measured (a) and tyrosinase inhibitory activities were determined (b). EC is *Ecklonia cava* and SS is *Sargassum siliquastrum*. Arbutin (20 mmol/L) and 1-phenyl-2-thiourea (PTU; 0.2 mmol/L) were utilized as positive controls. Concentration of the tested sample was 100 μg/mL. The experiments were conducted in triplicate and the data are expressed as mean ± standard error. Statistical evaluation was performed to compare the experimental groups and control groups. [a,b,c]Values having different superscripts are significantly different at $P < 0.05$.

Toxicity of melanogenic inhibitors in zebrafish embryo

In order to determine the toxicity of the melanogenic inhibitors, we monitored the growth patterns of zebrafish. The adopted experiment end-points used to assess the toxicity of the compounds included embryo mortality, morphological malformations and heartbeat disturbances. The melanogenic inhibitors (PTU, arbutin, EC and SS) were not associated with

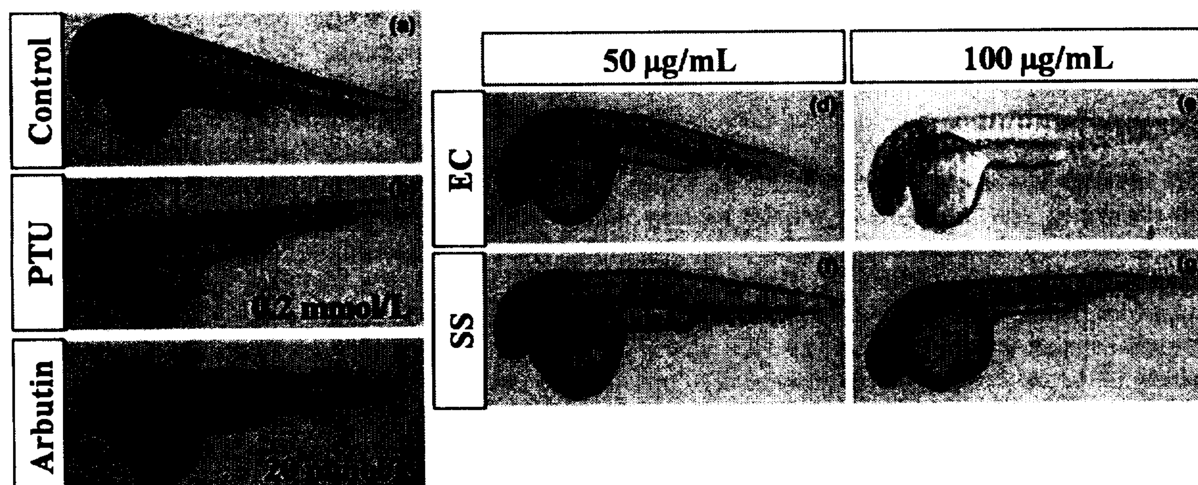

Figure 3. Effects of melanogenic inhibitors on the pigmentation of zebrafish embryos. Embryos were treated with melanogenic inhibitors at the indicated concentrations. The effects on the pigmentations of zebrafish embryos were observed by microscopy. (a) Untreated zebrafish, (b) 0.2 mmol/L 1-phenyl-2-thiourea (PTU), (c) 20 mmol/L arbutin, (d,e) in *Ecklonia cava* (EC)-treated zebrafish, (f,g) *Sargassum siliqustrum* (SS)-treated zebrafish. The image was observed at 35 high-power field (original magnificent ×20).

mortality in this experiment. When evaluating the morphological malformations, the inhibitors did not evidence conspicuous adverse effects (data not shown). On the other hand, in the heartbeat test, arbutin generated a slight disturbance, and PTU evidenced a marked increase in b.p.m., whereas EC and SS did not generate any heatbeat rate disturbances as compared with the controls (no treatment of inhibitor, Fig. 4). The zebrafish was allowed to continue to develop, because cosmetics are used daily. Interestingly, the PTU-treated zebrafish all died after the third day, and the arbutin-treated zebrafish survived at a rate of approximately 40% over the same period, whereas the algae extract-treated

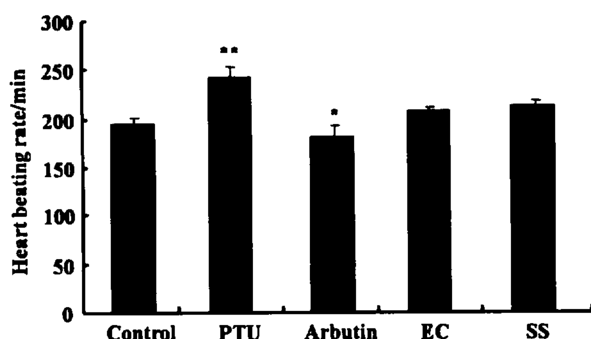

Figure 4. Effects of melanogenic inhibitors on the heartbeat rate for measurement of the toxicity of the tested sample. The heartbeat was measured at 35 days post-fertilization (dpf) by microscopy. The number of heartbeats over 3 min was counted, and the results are expressed as the b.p.m. Arbutin (20 mmol/L) and 1-phenyl-2-thiourea (PTU; 0.2 mmol/L) were used as the positive controls. Concentration of the tested sample was 100 μg/mL. The experiments were conducted in triplicate and the data are expressed as mean ± standard error. Statistical evaluation was performed to compare the experimental groups and control groups. $^{*}P < 0.05$ or $^{**}P < 0.01$.

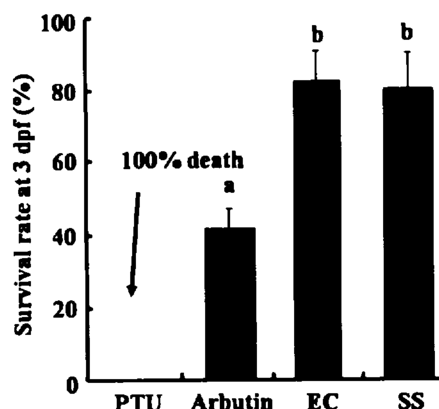

Figure 5. Effects of melanogenic inhibitors on survival of zebrafish after 3 days. The inhibitors exposed from 9–72 days post-fertilization (dpf) (63 h exposure). EC is *Ecklonia cava* and SS is *Sargassum siliquastrum*. Arbutin (20 mmol/L) and 1-phenyl-2-thiourea (PTU; 0.2 mmol/L) were utilized as positive controls. Concentration of the tested sample was 100 μg/mL. [a,b]Values having different superscripts are significantly different at $P < 0.05$.

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zebrafish showed a survival rate of approximately 80% (Fig. 5).

DISCUSSION

In the previous studies, a variety of materials have been developed and are currently utilized as cosmetic additives or as medicinal products for the treatment of hyperpigmentation.[25,26] Recently, the demand for natural products that inhibit or prevent skin pigmentation is increasing all over the world. A variety of natural or synthetic substances are currently utilized as ingredients of preparations designed to control hyperpigmentation, but none of these have proven completely satisfactory, either due to their limited efficacy or owing to safety concerns.[27] For instance, hydroquinone, which was used widely and until recently was considered the standard depigmenting agent, has now been banned for cosmetic uses in Europe and some Asian countries, and is available only by prescription. Kojic acid, another tyrosinase inhibitor, has a high sensitizing potential and has also been prohibited in some countries, citing mutagenic concerns.[28] Arbutin, a natural compound, is used extensively in the cosmetic industry as a response to increasing global demand for skin-whitening agent substances for the development of new depigmenting, cosmeceutical and skin lighting agents.[29,30] Marine algae have been used in food for centuries; they are frequently utilized to maintain good health or to treat a variety of diseases. In this study, these materials were screened for their tyrosinase inhibitory activities in order to confirm their cosmeceutical value (Table 1). The cosmetic potential of those extracts was assessed in primary investigations, and two extracts (EC and SS) were used in subsequent experiments. Therefore, we evaluated their inhibitory effects on tyrosinase and melanin synthesis in both a cell line and a zebrafish animal.

Tyrosinase is an important constituent of cosmetics and a known skin-lightening agent.[25] Pigment synthesis involves the conversion of tyrosine to melanin synthesis in melanocytes.[31] We utilized L-tyrosine as the substrate for the detection of this tyrosinase inhibitory effect in the cells. Among the extracts, the two species EC and SS have been shown to exert potent tyrosinase inhibitory effects (Table 2, Fig. 1). Kojic acid and arbutin function as whitening agents and evidenced tyrosinase inhibitory activity.[32,33] The tested extracts evidenced tyrosinase inhibitory activities similar to those of arbutin. Melanin formation is the most important determining factor in mammalian skin color.[34] In melanogenesis, the proximal pathway consists of the enzymatic oxidation of L-tyrosine or L-3,4-dihydroxyphenylalanine (L-DOPA) to its corresponding *o*-dopaquinone catalyzed through tyrosinase.[35] Marine algal extracts and their bioactive constituents have been previously explored with regard to tyrosinase inhibitory activity. According to Kang *et al.*,[36] *Ecklonia stolonifera* showed inhibitory activity with an IC_{50} value of 345 μg/mL. By way of contrast, our new findings reveal that inhibitors evidenced tyrosinase inhibitory effects that were dramatically more profound than those of *E. stolonifera*.

Kojic acid has been banned in some countries owing to mutagenic concerns and arbutin is a natural product, but it is a glucosylated hydroquinone and may pose similar risks of cancer.[37] In the current study, murine melanocytes were utilized to assess melanin synthesis inhibitory activity. Although, melanin synthesis inhibition of EC and SS was less profound than those observed in the positive controls, both EC and SS reduced cellular melanin content (Fig. 2). To exclude the possibility that the above inhibitory effects of EC, SS and arbutin on melanogenesis might be caused by the inhibition of cell growth, we compared the number of cells grown in the presence and absence of EC, SS and arbutin. EC, SS and arbutin did not seem to inhibit cell viability at a concentration below 100 μg/mL. These results indicate that EC, SS and arbutin had an inhibitory effect on melanogenesis at non-cytotoxic concentrations.

Despite its successful applications, the cell model has some disadvantages in terms of its physiological and economic relevance. For example, the data acquired from *in vitro* studies using cultured cells may not be directly extrapolated from the *in vivo* results. Clearly, *in vivo* tests using animal models or humans are the most physiologically relevant, but these tests are expensive, labor intensive and tedious, and require large amounts of precious compounds, particularly during the screening and compound evaluation steps. Furthermore, pressure to limit the use of animals is increasing, except for tests of preclinical toxicity and safety assessments.[38] Thus, in this study, we proposed a zebrafish model as an *in vivo* animal

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model for evaluation of the melanogenic regulatory compounds from marine algae. The value of the zebrafish as an animal model for drug discovery and toxicological studies has been recently recognized.[17,18] Additionally, the zebrafish has melanin pigmentations on its surface, allowing simple observation of the pigmentation process without the need for any complicated experimental procedures. The characteristic external pigmentation pattern of the zebrafish is generated by an array of three types of pigment cells, all of which are derived from the neural crest. These include melanophores (melanin-containing melanocytes), xanthophores (containing yellow pigment) and iridophores (containing reflecting platelets).[39] The combination of xanthophores and iridophores generates the yellowish-silver interstripes, whereas melanophores contribute to the formation of the characteristic longitudinal dark stripes of the epidermis.[40,41] Recognizing the advantages of this model, which include a rapid pigmentation process, permeability to small molecules and ease handling, we propose that zebrafish can be employed as a phenotype-based model for the screening of melanogenic regulatory compounds.[20] Additionally, the toxicity of certain compounds can be simultaneously determined by assessing the morphological malformations and heartbeat disturbance.[38]

In this study, we also evaluated the feasibility of zebrafish as an animal model system to determine the effects of marine algae for melanogenic inhibition. PTU is a sulfur-containing tyrosinase inhibitor which has been extensively utilized in zebrafish research as a pigment inhibitor.[24,42] Arbutin was also utilized as a positive control. All of the tested samples exerted profound inhibitory effects on zebrafish pigmentation, most likely as the consequence of their tyrosinase activity-inhibitory potential (Figs 2,3).

The zebrafish in this study were allowed to continue to develop, because cosmetics are used daily. Interestingly, the PTU-treated zebrafish were all dead after 3 days, and the arbutin-treated fish survived at a rate of approximately 40% over the same period, whereas the algae extract-treated zebrafish evidenced a survival rate of approximately 80%. Although both algae showed cytotoxicity at a relatively lower dose than the positive control, it did not inhibit cell viability at the concentration used in the study. However, positive controls showed high toxicity in *in vivo* experiment (Figs 4,5). In simultaneous *in vivo* toxicity tests, toxicity was not detected in the fish treated with either algal species, whereas toxicity was observed in the fish treated with the positive controls. Additionally, marine algae, EC and SS, have been shown in a previous study to possess excellent antioxidant activities and high phenolic content,[12] thus making them potential candidates for cosmetic application.

In summary, two marine algae – EC and SS – were evaluated in regard to their potential efficacy as skin-whitening agents, and they evidenced profound inhibitory effects against tyrosinase and melanin synthesis in both *in vitro* cell experiments and zebrafish animal model. It can be surmised that these algae are likely to be useful to the cosmetic and medicinal industries.

ACKNOWLEDGMENT

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (no. 2009-0071296).

REFERENCES

1 Shiino M, Watanabe Y, Umezawa K. Synthesis of N-substituted N-nitrosohydroxylamines as inhibitors of mashroom tyrosinase. *Bioorg Med Chem* 2001; **9**: 1233–1240.

2 Cabanes J, Chazarra S, García-Carmona F. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J Pharm Pharmacol* 1994; **46**: 982–985.

3 Shimogaki H, Tanaka Y, Tamai H, Masuda M. *In vitro* and *in vivo* evaluation of ellagic acid on melanogenesis inhibition. *Int J Cosmet Sci* 2000; **22**: 291–303.

4 Kim DS, Kim SY, Chung JH, Kim KH, Eun HC, Park KC. Delayed ERK activation by ceramide reduces melanin synthesis in human melanocytes. *Cell Signal* 2002; **14**: 779–785.

5 Shimizu K, Kondo R, Sakai K, Lee SH, Sato H. The inhibitory components from *Artocarpus incisus* on melanin biosynthesis. *Planta Med* 1998; **64**: 408–412.

6 Son SM, Moon KD, Lee CY. Rhubarb juice as a natural antibrowning agent. *J Food Sci* 2000; **65**: 1288–1289.

7 Xu Y, Stokes AH, Freeman WM, Kumer SC, Vogt BA, Vrana KE. Tyrosinase mRNA is expressed in human substantia nigra. *Brain Res Mol Brain Res* 1997; **45**: 159–162.

8 Noseda MD, Tulio S, Duarte MER. Polyscchharides from the red seaweed *Bostrychia montagnei*: chemical characterization. *J Appl Phycol* 1999; **11**: 35–40.

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9 Barbarino E, Lourenco SO. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. *J Appl Phycol* 2005; **17**: 447–460.
10 Kuda T, Tsunekawa M, Goto H, Araki Y. Antioxidant properties of four edible algae harvested in the Noto Peninsula, Japan. *J Food Compost Anal* 2005; **18**: 625–633.
11 Heo SJ, Jeon YJ. Radical scavenging capacity and cytoprotective effect of enzymatic digests of *Ishige okamurae*. *J Appl Phycol* 2008; **20**: 1087–1095.
12 Heo SJ, Cha SH, Lee KW, Cho KSM, Jeon YJ. Antioxidant activities of chlorophyta and phaeophta from Jeju Island. *Algae* 2005; **20**: 251–260.
13 Eisen JS. Zebrafish make a big splash. *Cell* 1996; **87**: 969–977.
14 Fishman MC. Zebrafish genetics: the enigma of arrival. *Proc Natl Acad Sci USA* 1999; **96**: 10554–10556.
15 Driever W, Solnica-Krezel L, Schier AF. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 1996; **123**: 37–46.
16 Kimmel CB. Genetics and early development of zebrafish. *Trends Genet* 1989; **5**: 283–288.
17 den Hertog J. Chemical genetics: drug screens in zebrafish. *Biosci Rep* 2005; **25**: 289–297.
18 Pichler FB, Laurenson S, Williams LC, Dodd A, Copp BR, Love DR. Chemical discovery and global gene expression analysis in zebrafish. *Nat Biotechnol* 2003; **21**: 879–883.
19 Langheinrich U. Zebrafish: a new model on the pharmaceutical catwalk. *Bioessays* 2003; **25**: 904–912.
20 Choi TY, Kim JH, Ko DH *et al*. Zebrafish as a new model for phenotyoe-based screening of melanogenic regulatory compounds. *Pigment Cell Res* 2007; **20**: 120–127.
21 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
22 Kim YM, Yun J, Lee CK, Lee H, Min KR, Kim Y. Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosinase and mechanism of action. *J Biol Chem* 2002; **277**: 16340–16344.
23 Son AR, Choi JC, Kim JA *et al*. Isolation of melanogenesis inhibitors from ponciri fructus. *Kor J Pharmacogn* 2005; **36**: 1–8.
24 Karlsson J, von Hofsten J, Olsson PE. Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development. *Mar Biotechnol* 2001; **3**: 522–527.
25 An BJ, Kwak JH, Park JM *et al*. Inhibition of enzyme activities and the antiwrinkle effect of polyphenol isolated from the persimmon leaf (*Diospyros kaki folium*) on human skin. *Dermatol Surg* 2005; **31**: 848–854.
26 Wang Q, Qiu L, Chen XR, Song KK, Shi Y, Chen QX. Inhibitory effects of phloridzin dihydrate on the activity of mushroom (*Agaricus bisporus*) tyrosinase. *Bioorg Med Chem* 2007; **15**: 1568–1571.
27 Briganti S, Camera E, Picardo M. Chemical and instrumental approaches to treat hyperpigmentation. *Pigment Cell Res* 2003; **16**: 101–110.
28 Petit L, Pierard GE. Analytic quantification of solar lentigines lightening by a 2% hydroquinone-cyclodextrin formulation. *J Eur Acad Dermatol Venereol* 2003; **17**: 546–549.
29 Seo SY, Sharma VK, Sharma N. Mushroom tyrosinase: recent prospects. *J Agric Food Chem* 2003; **51**: 2837–2853.
30 Aburjai T, Natsheh FM. Plants used in cosmetics. *Phytother Res* 2003; **17**: 987–1000.
31 Li YP, Hsu FL, Chen CS, Chern JW, Lee MH. Constituents from the Formosan apple reduce tyrosnase activity in human epidermal melnocytes. *Phytochemistry* 2007; **68**: 1189–1199.
32 Nihei KI, Kubo I. Identification of oxidation product of arbutin in mushroom tyrosinase assay system. *Bioorg Med Chem Lett* 2003; **13**: 2409–2412.
33 Roh JS, Han JY, Kim JH, Hwang JK. Inhibitory effects of active compounds isolated from Safflower (*Carthamus tinctorius* L.) seeds for melanogenesis. *Biol Pharm Bull* 2004; **27**: 1976–1978.
34 Hearing VJ. Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J Dermatol Sci* 2005; **37**: 3–14.
35 Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell Mol Life Sci* 2005; **62**: 1707–1723.
36 Kang HS, Chung HY, Kim JY, Son BW, Jung HA, Choi JS. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. *Arch Pharm Res* 2004; **27**: 194–198.
37 O'Donoghue JL. Hydroquinone and its analogues in dermatology – a risk-benefit viewpoint. *J Cosmet Dermatol* 2006; **5**: 196–203.
38 Zon LI, Peterson RT. *In vivo* drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005; **4**: 35–44.
39 Jin EJ, Thibaudeau G. Effects of lithium on pigmentation in the embryonic zebrafish (*Brachydanio rerio*). *Biochim Biophys Acta* 1999; **1449**: 93–99.
40 Hirata M, Nakamura K, Kondo S. Pigment cell distributions in different tissues of the zebrafish, with special reference to the striped pigment pattern. *Dev Dyn* 2005; **234**: 293–300.
41 Kelsh RN, Brand M, Jiang YJ. Zebrafish pigmentation mutations and the processes of neural crest development. *Development* 1996; **123**: 369–389.
42 Elsalini OA, Rohr KB. Phenylthiourea disrupts thyroid function in developing zebrafish. *Dev Genes Evol* 2003; **212**: 593–598.

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