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A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Isolation and Identification of Marine Cosmeceutical Whitening Compounds from Brown Seaweeds

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GRADUATEDSCHOOL

JEJU NATIONAL UNIVERSITY

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Isolation and Identification of Marine Cosmeceutical Whitening Compounds from Brown Seaweeds

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국문초록

멜라닌은 인체 피부와 모발의 주요 색깔의 결정에 관여하는 색소 성분으로서 이러한 멜라닌의 합성과정은 멜라닌 세포에 존재하는 멜라노좀에서 합성되며, 이러한 과정은 tyrosinase나 tyrosinase-relative protein 1, 2 (TRP-1, TRP-2) 그리고 MAPK 등에 의해 조절된다.

본 연구에서는 제주 연안에 서식하고 있는 17종의 갈조류를 대상으로 tyrosinase 저해 활성 및 멜라닌 합성 저해 효과를 screening한 결과 넓패 (*Ishige foliacea*), 바위수염(*Myelophycus caespitosus*), 지충이 (*Sargassum thunbergii*), 참가죽그물바탕말 (*Dictyota coriacea*) 그리고 패 (*Ishige okamurae*)에서 다른 갈조류에 비해 우수한 효과를 나타내는 것을 확인하였고, raw material 확보여부 등을 고려하여 넓패를 대상으로 활성물질을 분리하였다.

분리된 미백활성 물질인 OPA로 tyrosinase 저해 활성을 측정한 결과 IC 50 값이 14.9±0.03으로 낮은 농도에서도 우수한 저해활성을 나타내었다. 이에 따라 멜라닌을 생성하는 흑색종 세포주인 B16F10 세포에서 α-MSH 자극으로 유발된 멜라닌 합성에 대한 영향을 알아본 결과 50 μM의 농도 내에서 OPA의 독성의 거의 나타나지 않았으며 12.5-50 μM까지

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농도의존적으로 멜라닌 합성이 억제되었으며 더불어 tyrosinase의 활성도 농도의존적으로 저해되었다. 뿐만 아니라 시판되는 미백 활성물질인 arbutin 보다 우수한 저해 활성을 나타내는 것을 확인하였다. Western blotting에서 OPA는 tyrosinase, TRP-1, TRP-2, MITF의 발현을 농도에 비례하여 억제하였으며, MAPK활성화에 미치는 영향을 조사한 결과 ERK, Akt, JNK가 인산화되고 p38의 활성을 억제함으로써 melanin 합성이 지연되는 것을 확인할 수 있었다. 또한 기능성 소재탐색을 위한 실험 대체동물로 각광받고 있는 in vivo 모델인 zebrafish 실험을 통한 melanin contents 및 tyrosinase 저해 활성에서도 우수한 효과를 나타냄을 확인할 수 있었다.

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INTRODUCTION

The oceans cover more than 70% of the Earth's surface with marine species comprising approximately half of the total global biodiversity. Therefore the wide diversity of marine organisms is being recognized as rich sources of functional materials. Marine organisms, marine algae are still identified as an under-exploited plant resource although they have long been used in food diets as well as traditional medicine in Asian countries such as Japan, China and Korea.Among them brown seaweeds have the brown color resulted from the dominance of the fucoxanthin pigment and various polysaccharides, and possess many kinds of bioactive properties.

Polyphenolic compounds are one of the most common classes of secondary metabolites found in terrestrial plants and marine algae. There are fundamental differences in the chemical structures of polyphenols in both terrestrial and marine plants (Shibata et al., 2002). In general, polyphenols of phenolic compounds have a similar basic structural chemistry including an 'aromatic' or phenolic' ring structure. Phenolic compounds have been associated with antioxidative action in biological systems, acting as scavengers of single oxygen and free radicals (Rice-Evans et al., 1995; Jorgensen et al., 1999). The protective effects of plant polyphenols in biological

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systems are ascribed to their capacity to transfer electrons to free radicals, chelate metal catalysis, activate antioxidant enzymes and inhibit oxidase. Polyphenols are classified broadly into two classes; condensed tannins, which are polymetric flavonoids, and hydrolysable tannins, which are derivatives of gallic acid (Haslam, 1989). Phlorotannins, marine algal polyphenols, consisting of phloroglucinol units linked to one another in various ways, occur broadly among the brown and red algae (Singh and Bharate, 2006). Several researches on those kinds of compounds have pointed out a variety of biological benefits including antioxidant, anticoagulant, antihypertension, antibacterial and antitumor activities (Nagayama et al., 2002; Mayer and Hamann, 2005; Athukorala and jeon, 2005; Kotake-Nara et al., 2005; Heo et al., 2008).

Melanin is the most important pigment for humans. It is the major pigment for the color of human skin and hair (Hearing, 1999). Human skin exists in a wide range of different colors and gradations, ranging from white to brown to black. This is due to the presence of a chemically inert and stable pigment known as melanin, which is produced deep inside the skin but is displayed as a mosaic at the surface of the body. Melanin is therefore responsible for the most striking polymorphic traits of humans and for the most obvious and thoroughly discussed aspect of human geographical variability: skin color. Besides its role in defining ethnicity, melanin plays an essential role in defending

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the body against harmful UV rays and other environmental challenges (Costin et al., 2007). In addition to it determines our race and phenotypic appearance. But it may be overproduced with chronic sun exposure, melisma or other hyperpigmentation disease (Gupta et al., 2006). And the accumulation of an abnormal melanin amount in different specific parts of the skin as more pigmented patches (melasma, freckles, ephelide, senile lentigines etc.) might become an esthetic problem yet. In Western countries, skin lighteners are applied for the prevention and treatment of irregular hyperpigmentation, such as melasma, freckles or age spots. However, in Asia, traditional fork herbs have been widely used as skin whitening agents (Solano et al., 2006).Therefore, we need depigmenting. Skin pigmentation abnormalities include natural and synthetic depigmentation agents can be minimized by the action of safe and effective regulators.

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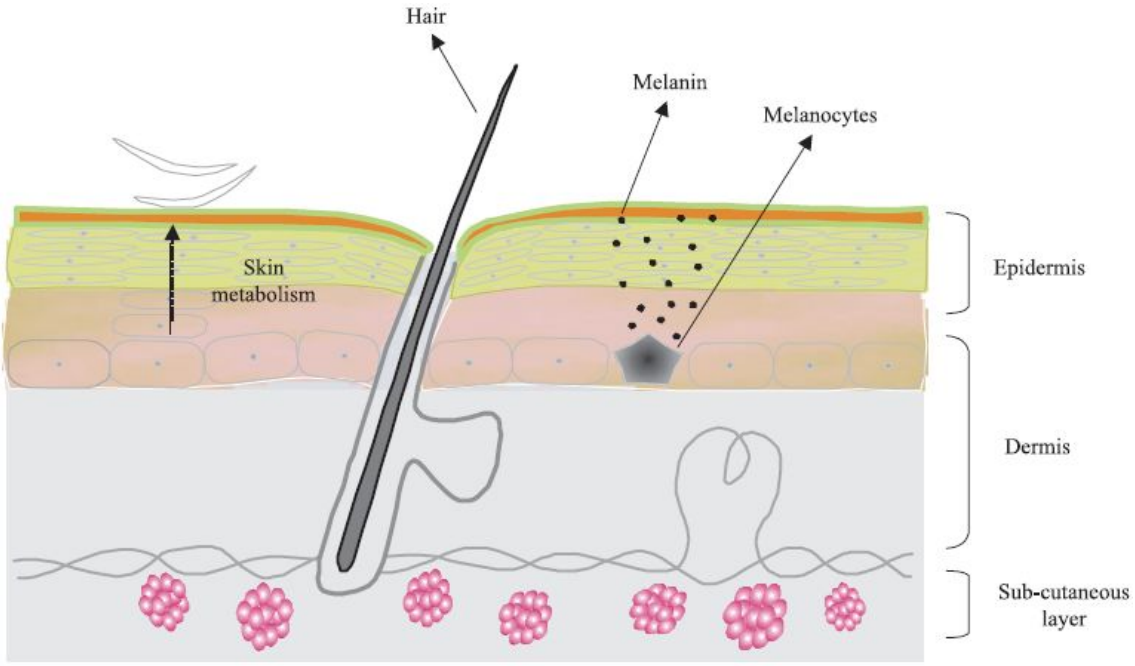

Figure Ⅰ. Outline of mammalian skin indicating three main layers epidermis, dermis and sub-cutaneous, visible pigmentation results from the synthesis and distribution of melanin in the melanocytes in the dermis layer of the skin.

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Tyrosinase is a multifunctional copper-containing enzyme and commonly present in microorganisms, plants and animals. Tyrosinase is responsible for enzymatic browning in plants, and it may cause undesirable changes in color, flavor and nutritive values of plant-derived foods and beverages (Sanchez-Ferrer et al., 1995). This enzyme is mainly involved in the initial steps of the pathway which consist to the gydroxylationof the p-monophenolic amino acid 1—tyrosine (monophenolase activity of tyrosinase) and the oxidation of the product of this reaction, the o-diphenolic amino acid L-DOPA (diphenolase activity), to give rise to o-dopaquinine (Baurin et al., 2002) (**Figure II**).

Three major melanosome accessory enzymes of the tyrosinase family are involved in melanin biosynthesis. Tyrosinase, the rate-limiting enzyme, catalyzes two distinct reactions of melanin synthesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone (Tripathi et al., 1992). In the absence of thiol-containing compounds, dopaquinone spontaneously converts initially to dopachrome and then to indole-5,6- quinone or indole-5,6-quinone 2-carboxylic acid. Subsequently tyrosinase-related protein-2 (TRP2/DCT; dopachrome tautomerase) and tyrosinase related protein-1 (TRP1; DHICA oxidase) act to produce unstable quinones that undergo further polymerization yielding melanin (Palumbo et al., 1991; Mallick et al., 2005).

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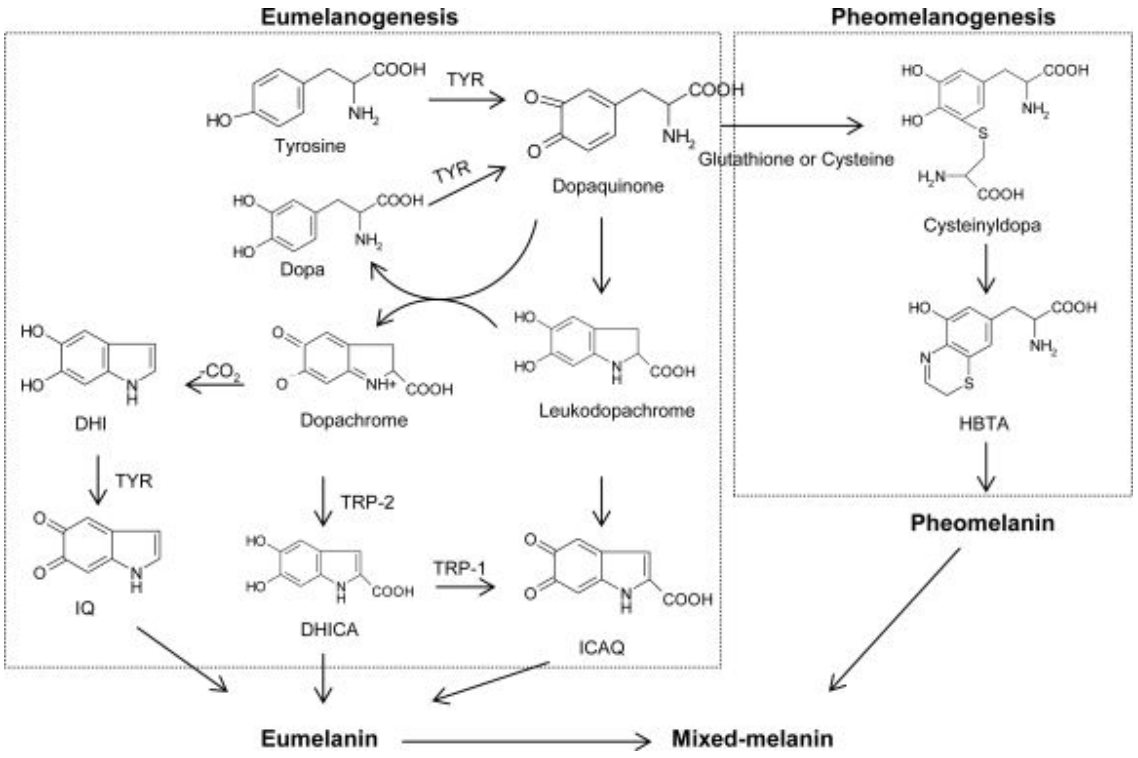

Figure Ⅱ. Biosynthetic pathway of melanin. TYR, tyrosinase; TRP; tyrosinase related protein; dopa, 3,4 dihydroxyphenylalanine; DHICA, 5,6 dihydroxyindole 2 carboxylic acid; DHI, 5,6 dihydroxyindole; ICAQ, indole 2 carboxylic acid 5,6 quinone; IQ, indole 5,6 quinone; HBTA, 5 hydroxy 1,4 benzothiazinylalanine.

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Tyrosinase is the prime regulatory enzyme in the melanin synthetic pathway (Tachobana et al., 2000), and its promoter is a principal target of the microphthalmia-associated transcription factor (MITF). Early studies (Bentley et al., 1994; Yasumotoet al, 1994) have identified MITF as the major transcriptional regulator of tyrosinase and pigmentation. MITF gene contains two promoters, one of which bears a cAMP-responsive element (CRE). Phosphorylation of a cAMP-responsive element binding protein (CREB) activity is needed for intact CRE to initiate MITF expression (Price et al., 1998; Fuse et al., 1996; Goding, 2000; Kadekaro et al., 2003).

The cellular response to extracellular stimuli such as chemicals is mediated by several intracellular signal transduction pathways (Hunter, 1995) including cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway; mitogen activated protein kinase (MAPK) cascade reaction; diacylglycerol/protein kinase C (DAG/PKC) pathway; and phosphatidylinositol 3-kinase/protein kinase B (PI-3K/PKB) pathway.

The cAMP-triggered mechanisms on melanogenesis have been investigated in earlier studies: cAMP/PKA pathway, through phosphorylation of CREB, increase the expression of MITF, and subsequently up-regulates the tyrosinase expression, and the classical agents are α-MSH and forskolin (Bertolotto et al., 1998); it has also been shown that cAMP-independent PKA pathway (Hemesath et al., 1998; Wu et al., 2000) mediates regulation of tyrosinase expression and melanogenesis via MITF.In addition to

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cAMP signalingpathway, MAPK cascade reaction is another important signaling transduction system in regulation of melanin synthesis (**Figure Ⅲ**). MAPKs include extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt p38 MAPKs, and c-jun N-terminal or stress-activated protein kinase (JNK/SAPK). It was reported that ERK (Kim et al., 2005; Choi et al., 2005; Kim et al., 2007) and AKT (Oka M et al., 2000), p38 MAPK(Saha et al., 2006; Tada et al., 2002; Smalley and Elisen., 2000; Mansky et al., 2002) mediated regulation of tyrosinase expression and melanogenesis via CREB to MITF. The PKC signaling pathway is also involved in the regulation of melanin synthesis (Friedmann et al., 1990). These researches suggest that melanogenesis is a complex physiological function regulated by extracellular stimuli via various intercellular signal transduction pathways.Thus substances that inhibit such as tyrosinase, TRPs, MITF, ERK, Akt, p38 and JNK may be incorporated into cosmetic preparations.

Recently, a number of studies have been focused on marine bio-resources. Marine natural products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agents. In this study, we tried to screen active compounds from 17 brown seaweeds and examined cosmeceutical activities of the active compounds which can be developed as possible cosmetic agents for human skin.

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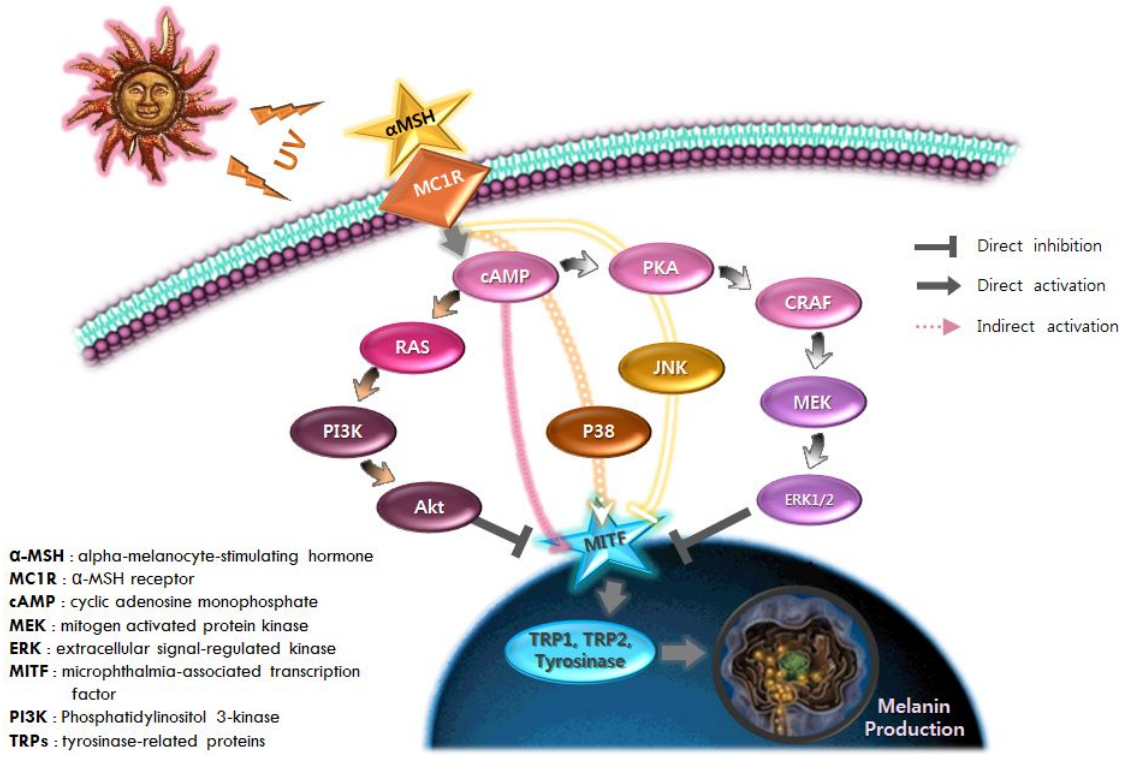

Figure Ⅲ. Model of contribution of MAPK signal transduction in melanogenesis.

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Zebrafish (Daniorerio) is small tropical fish native to Southeast Asia. They have a unique combination of genetic and experimental embryologic advantages that make them ideal for studying early development, particularly the embryogenesis of the circulatory system. And they have emerged as a highly advantageous vertebrate model organism because of its small size, large clutches, transparency, low cost and physiological similarity to mammals (Eisen, 1996; Fishman,1996).

Traditionally,zebrafish has been used in the fields ofmolecular genetics and developmental biology (Driever et al., 1996; Kimmel, 1989).Because genome analysis shows that 90% of all human genes have orthologs in zebrafish.However, its value as a model organism for drug discovery and toxicological studies has been recognizedrecently (Den Hertog, 2005; Pichler et al., 2003). The application of drugs and/or smallmolecules to zebrafish is simple because the early stage embryo rapidly absorbs small molecular compoundsdiluted 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 (Langheinrich, 2003).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

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process without complicated experimental procedures (Choi et al., 2007).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.

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Part Ⅰ.

Screening and purification of potential whitening compounds from brown seaweeds

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1. ABSTRACT

Whitening activities of 17 species of the brown seaweeds collected from coastal area of the Jeju Island were measured by tyrosinase inhibitory and melanin contents inhibitory effects. A variety of methanol extracts of brown seaweeds showed whitening activity. Among them, *Ishige foliacea, Myelophycus caespitosus, Sargassum thunbergii, Dictyota coriacea and Ishige okamurae* extract exhibited strong activities both tyrosinase inhibitory and melanin contents inhibitory effect. For the development of whitening agents for cosmeceutical bio-materials, we selected *I. foliacea*. Ethyl acetate (EtOAc) fractions of *I. foliacea* extracts showed higher whitening activities than the other organic solvent fractions. Therefore, the fractions of *I. foliacea* extracts were selected for use in further isolation of octaphlorethol A (OPA). The structure of OPA was elucidated based on NMR spectroscopic data.

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2. Materials and methods

2.1. General experimental procedures

The UV and FR-IR spectra were recorded on a pharmacia Biotech Ultrospec 3000 UV/Visible spectrometer and a SHIMAZU 8400s FT-IR spectrometer, respectively. NMR spectra were recorded on a Varian INOVA 400 MHz spectrometer, respectively. The preparative HPLC was carried out on a DIONEX prep-HPLC system and Chromeleon software using C18 column (uBondapac RP-18, 19× 300 mm, 10 μm, Waters Co.). The HPLC was carried out on a YoungLin Instrument HPLC system equipped with a YoungLin acme 9000 UV/VIS detector and Autochrome software using C18 column (Waters Spherisorb® DOS-2 RP-18, 4.6× 250 mm, 5 μm, Waters Co.).

2.2. Materials

Seventeen species of brown seaweeds were collected along the coast of Jeju island, Korea, between March 2009 and March 2011. The samples were washed three times

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with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at -20℃. The frozen samples were lyophilized and homogenized with a grinder prior to extraction. Mushroom tyrosinase were purchased form Sigma Chemical Co. (St. Louis, MO, USA). And other chemical used were 99% or greater purity.

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Table 1-1.The list of polyphenol content of brown seaweeds.

No.	Korean name	Scientific name	Polyphenol content (g/100g)
1	넓패	*Ishige foliacea*	42.8
2	미역	*Undaria pinnatifida*	14.5
3	바위수염	*Myelophycus caespitosus*	40.2
4	부챗말	*Padina arborescens*	44.5
5	지충이	*Sargassum thunbergii*	23.9
6	참가죽그물바탕말	*Pachydictyon coriaceum*	10.4
7	패	*Ishige okamurae*	53.8
8	톳	*Hijikia fusiforme*	13.4
9	짝잎모자반	*Sargassum hemiphyllum*	11.4
10	괭생이모자반	*Sargassum horneri*	25.1
11	구슬모자반	*Sargassum piluiferum*	13.9
12	큰잎모자반	*Sargassum ringgoldianum*	67.9
13	몽당잎모자반	*Sargassum muticum*	13.1
14	쌍발이모자반	*Sargassum patens*	25.3
15	외톨개모자반	*Myagropsis myagroides*	15.7
16	큰열매모자반	*Sargassum macrocarpum*	14.5
17	큰톱니모자반	*Sargassum giganteifolium*	38.5

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2.3. Preparation of 80% methanolic extracts from brown seaweeds

The brown seaweeds samples were pulverized into powder using a grinder. The algal powder (1 g) was extracted with 80% methanol (100 ml) at room temperature for 24h and filtrated. After filtration, the methanolic extracts were evaporated under vacuum to obtain dry samples. The dried extracts were used for further biological study.

2.4. Mushroom tyrosinase inhibition assay

Tyrosinase inhibitory activity was performed according to the method of Vanni et al., 1990. Each extract (10 µl) was diluted with 140 µl of 0.1 mM sodium phosphate buffer (pH 6.8) in a 96-well plate and then 40 µl of 1.5 mM L-tyrosine solution and 10 µl of mushroom tyrosinase (2100 units/ml) were added into a 96-well plate. The test mixture (200 µl) was mixed well and incubated at 37℃ for 12 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined by a multiplate reader at 490 nm against a blank, and the percent inhibition of tyrosinase activity was calculated using the following formula:

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

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A : Absorbance at 490 nm with test sample before incubation

B : Absorbance at 490 nm with test sample after incubation

C : Absorbance at 490 nm without test sample before incubation

D : Absorbance at 490 nm without test sample after incubation

2.5. Cell culture

B16F10 mouse melanoma cells were**(Fig 1-1)**obtained from KCLB (Korean cell line bank). The B16F10 cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (Fetal bovine serum) and 1% penicillin/streptomycin (100 X), and incubated at 37℃ under 5% CO_2 atmosphere. The cells were seeded (5 x 10^4) into 60 mm tissue culture dish, and sample treatment began 72h after seeding.

2.6. Measurement of cellular melanin contents

Cellular tyrosinase activity was measured using a previously described method (Tsuboi et al.,1998). B16F10 cells were treated with OPA for 3 days, were washed with

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PBS, and were then in 1 N NaOH in 10% DMSO at 80℃ for 1hr. The relative melanin content was determined by measuring the absorbance at 475 nm using an enzyme-linked immune sorbent assay (ELISA) reader.

2.7. Isolation and structural identification of octaphlorethol A (OPA)

The dried *Ishige foliacea*(**Fig. 1-2**)powder was extracted three times with 80% aqueous MeOH, and filtered. The filtrate evaporated at 40℃ to obtain the MeOH extract, which was dissolved in water, then partitioned with EtOAc. The EtOAc extract was fractionated by silica column chromatography with stepwise elution of $CHCl_3$-MeOH mixture (50:1-0:1) to afford separated active fractions. A combined active fraction was further subjected to prep-HPLC, and then finally purified reversed-phase HPLC to give novel compound Octaphlorethol A (OPA).

Octaphlorethol A (OPA): brownish yellow amorphous powder, ^{1}H NMR (400 MHz, DMSO-d_6), see **Table 1-2**; ESI-MS m/z 992.19 [M-2H]($C_{48}H_{34}O_{25}$: 994.14389).

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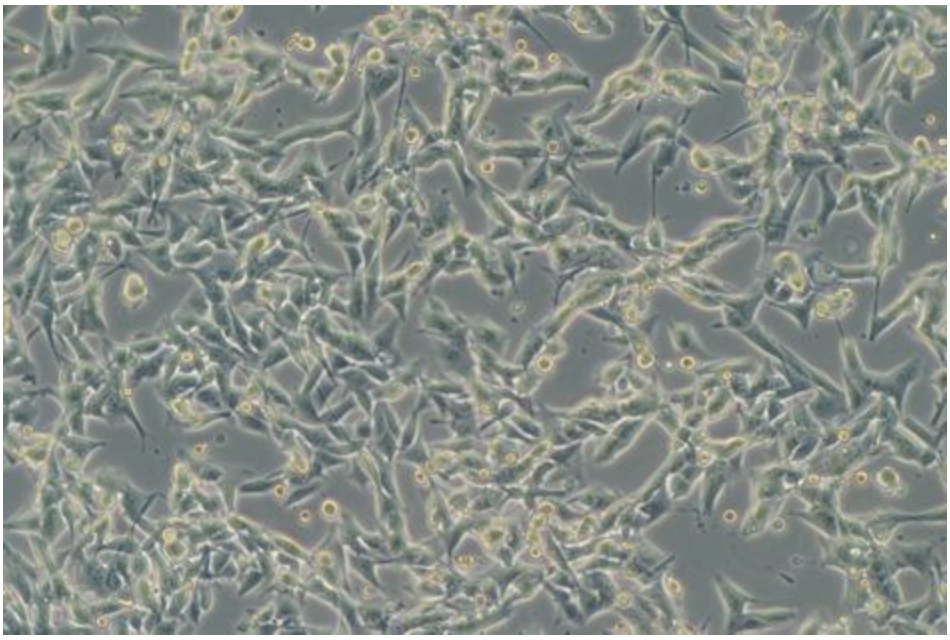

Fig. 1-1.The photography of B16F10 melanoma cells.

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Fig. 1-2.The photography of a brown alga *Ishige foliacea*.

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3. Results and Discussion

The initial purpose of the present study was to investigate the potential of brown seaweeds as hypopigmenting agents for cosmetic applications. Hyperpigmentation is associated with skin disease corresponds to the lung condition in Korean medical principle. In Asian countries, many women have problems with hyperpigmentation such as melisma and freckle (Oh et al., 2010). In addition, the light skin tone is an essential condition for beauty, so woman wants skin whitening. Therefore, recently, there has been growing interest in alternative cosmetic and in the therapeutic use of natural products for whitening especially those derived from herbs (Moon et al., 2010). This is because plant sources are usually considered to be less toxic with fewer side effects than synthetic ones. Marine algae are known to provide an abundance of bioactive compounds with great pharmaceutical foods and biomedical potential.

Brown seaweeds are abundant along the coast of Jeju Island and regarded as an edible algae. However since biological studies of this algae are relatively rare, this study screened its inhibitory effect of tyrosinase and melanin contents inhibitory effect.

In previous studies, we isolated dieckol and diphlorethohydroxycarmalol, a type of phlorotannin isolated from the brown seaweeds*Ecklonia cava* and Ishige okamurae of Jeju Island, Korea, and demonstrated its inhibitory effect of melanogenesis in *in*

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vitro(Heo et al., 2009; Wijesinghe., 2011).But there was no in case of studied deeply to signaling pathway. Therefore, in this study, we tried to screen active compounds from 17 species of brown seaweeds and evaluated depigmentation effect of the active compounds which can be used as possible functional cosmetics or nutraceuticalsfor whitening.

Tyrosinase is one of the important enzymes in the mammalian melanin synthesis. In the process of melanin synthesis, tyrosine is oxidized to DOPA (3,4-dihydroxyphenylalanine), and DOPA is further oxidized to DOPAquinone. Tyrosinase is an enzyme catalyzing this oxidation of tyrosine; therefore, chemicals that inhibit the activity of tyrosinase can be used as skin whitening agents (Kang et al., 2011). And total melanin content as a final product and the activity of tyrosinase, a key enzyme, in melanogenesis, were estimated (Hu, 2008).The inhibitory effect of tyrosinase and melanin contents by brown seaweeds extracts was presented in **Fig. 1-3**and **1-4**, respectively. It was observed whether brown seaweeds inhibited cellular melanin biosynthesis in B16F10 cells. *Ishige foliacea* (No. 1), *Myelophycus caespitosus* (No. 3), *Sargassum thunbergii* (No. 5), *Pachydictyon coriaceum* (No. 6) and*Ishige okamurae* (No. 7) extract suppressed α-MSH-induced melanogenesis involving the inhibition of melanin biosynthesis at most lower levels than control (**Fig, 1-3** and 1-**4**). A relationship between polyphenolic compound and melanogenesis inhibitory effect in marine algae

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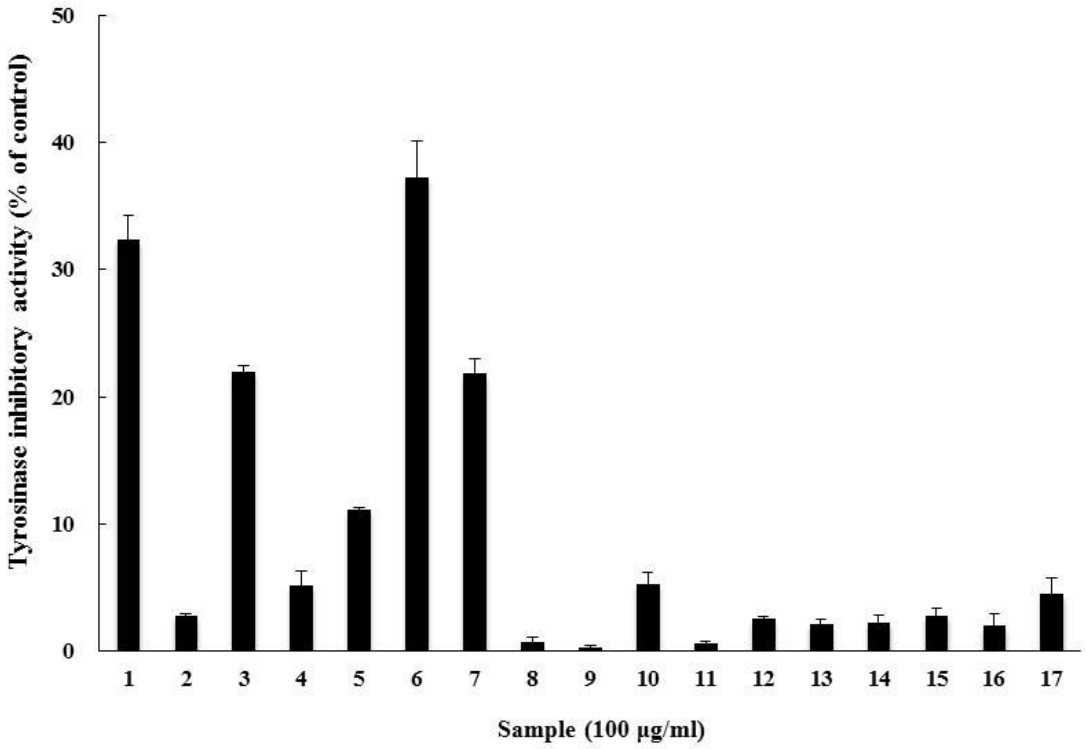

Fig. 1-3.Tyrosinase inhibitory effect of 80% MeOH extracts from brown seaweeds.

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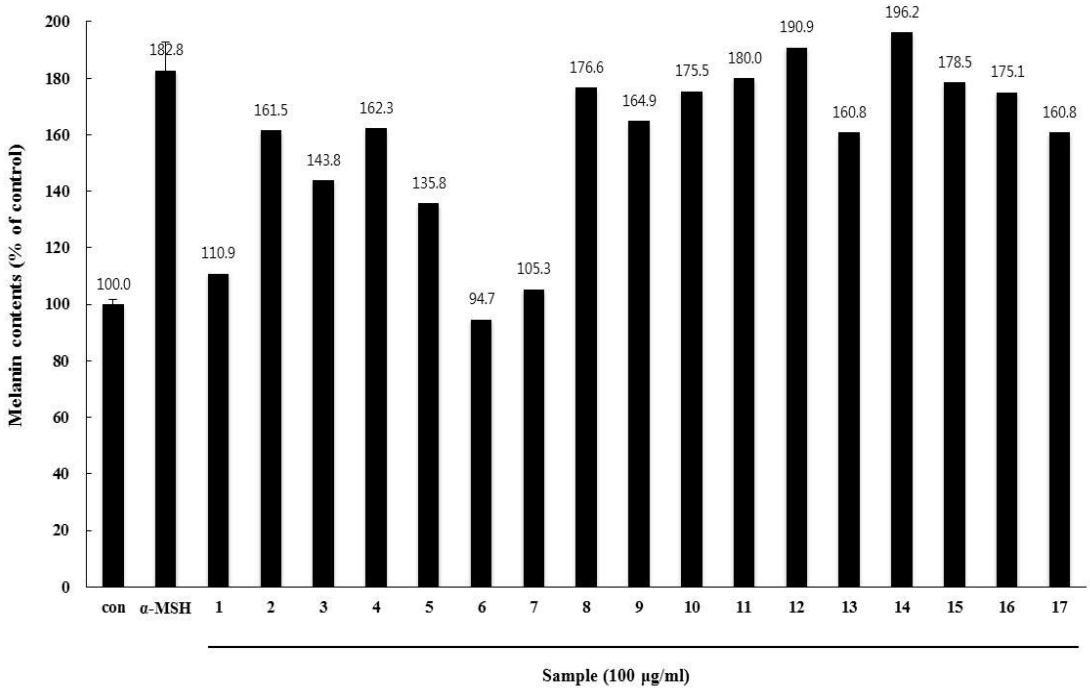

Fig. 1-4. Melanin contents of 80% MeOH extracts from brown seaweeds.

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has been reported (Heo et al., 2009; Heo et al., 2010). Polyphenolic compounds such as tannins from terrestrial plants and Phlorotannins from marine algae associate with a variety of proteins to form complexes. The results of several recent studies have demonstrated that the hydroxyl groups in polyphenolic compounds may, therefore, perform a crucial function in promoting inhibitory activity. Also, this study showed that brown seaweeds with high amount polyphenol displayed higher inhibitory activity (**Table 1-1**).

The *I. foliacea*, *M. caespitosus*, *S. thunbergii*, *P. coriaceum* and*I. okamurae* showed relatively higher effects both tyrosinase and melanogenesis inhibitory effect than the other brown seaweeds. For the development of whitening agents for cosmeceutical bio-materials, we investigated the inhibitory effect of seaweed extracts against tyrosinase and melanin contents. *I.foliacea*was selected as the target material on collection of raw materials and edibility. Therefore, the *I. foliacea* extract was selected for use in further experiment. The whitening effects of the organic solvent fractions on tyrosinase and melanogenesis inhibitory effect were shown in **Fig. 1-5**. The 80% methanol extracts were successfully partitioned according to their polarity. It was observed that EtOAc fraction exhibited the highest effects both tyrosinase and melanogenesis inhibitory effect compared to the other organic solvent fractions. Therefore, the fractions of *I.*

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foliacea extracts were selected for use in further isolation and purification process.

As the EtOAc extract showed prominent whitening effects, the active compound of this extract was fractionated by silica gel open column chromatography and prep-HPLC, and then finally novel active compound were purified by reversed-phase HPLC (**Fig. 1-6**). Octaphlorethol A (OPA) was isolated as a brownish yellow amorphous powder, and its molecular formula deduced as $C_{48}H_{34}O_{25}$ based on NMR (**Table 1-2 and Fig. 1-7, Fig. 1-8**) and ESI-MS analysis ($M\text{-}2H^2$, m/z: 994.14389) (**Fig. 1-9**). The OPA was then used in further experiments regarding on whitening agents for cosmeceutical bio-materials.

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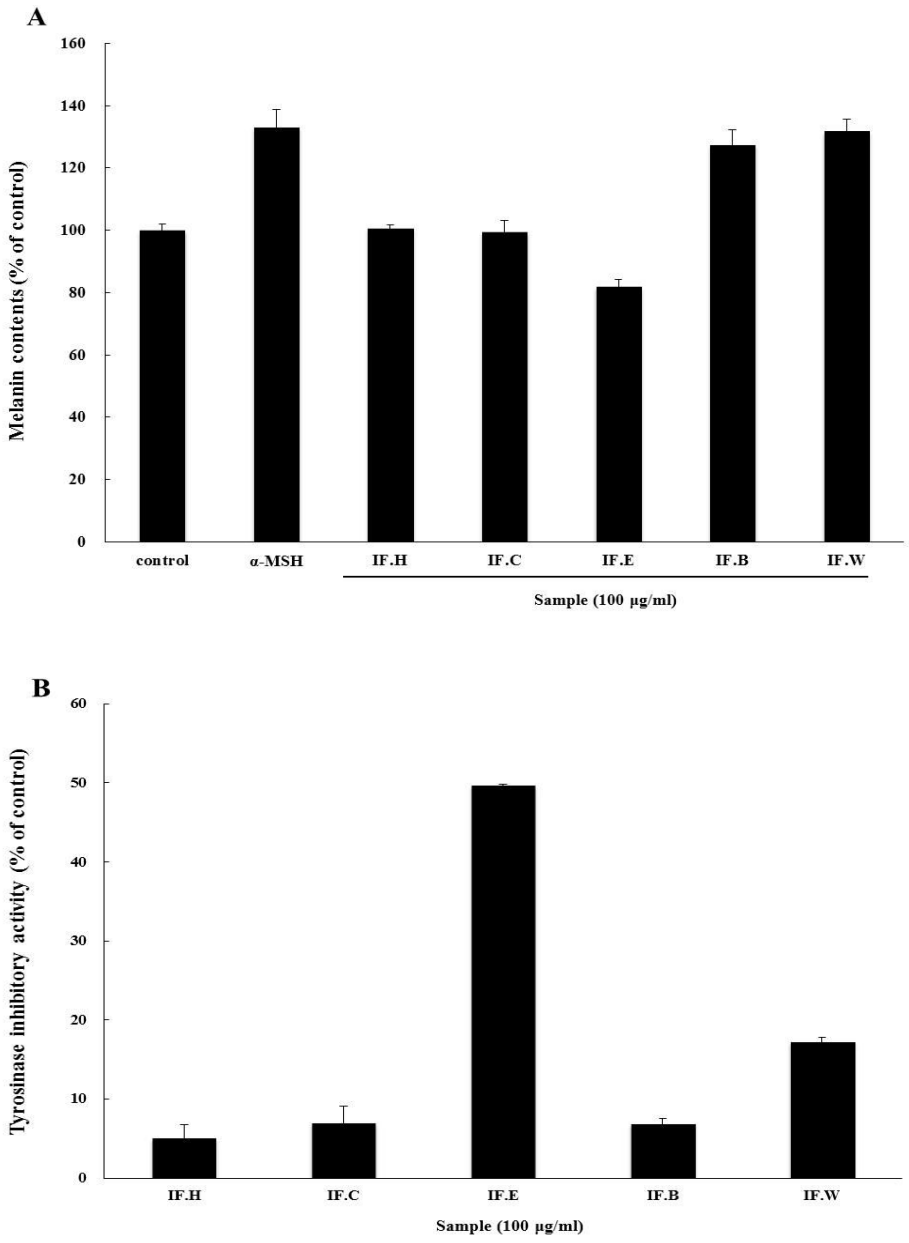

Fig. 1-5.Cellular melanin synthesis (A) and tyrosinase inhibitory effect (B) of I. foliacea extracts partitioned by various organic solvents.IF.H, Hexane fraction; IF.C, Chloroform fraction; IF.E, Ethyl acetate fraction; IF.B, Buthanol fraction; IF.W, Distilled water fraction.

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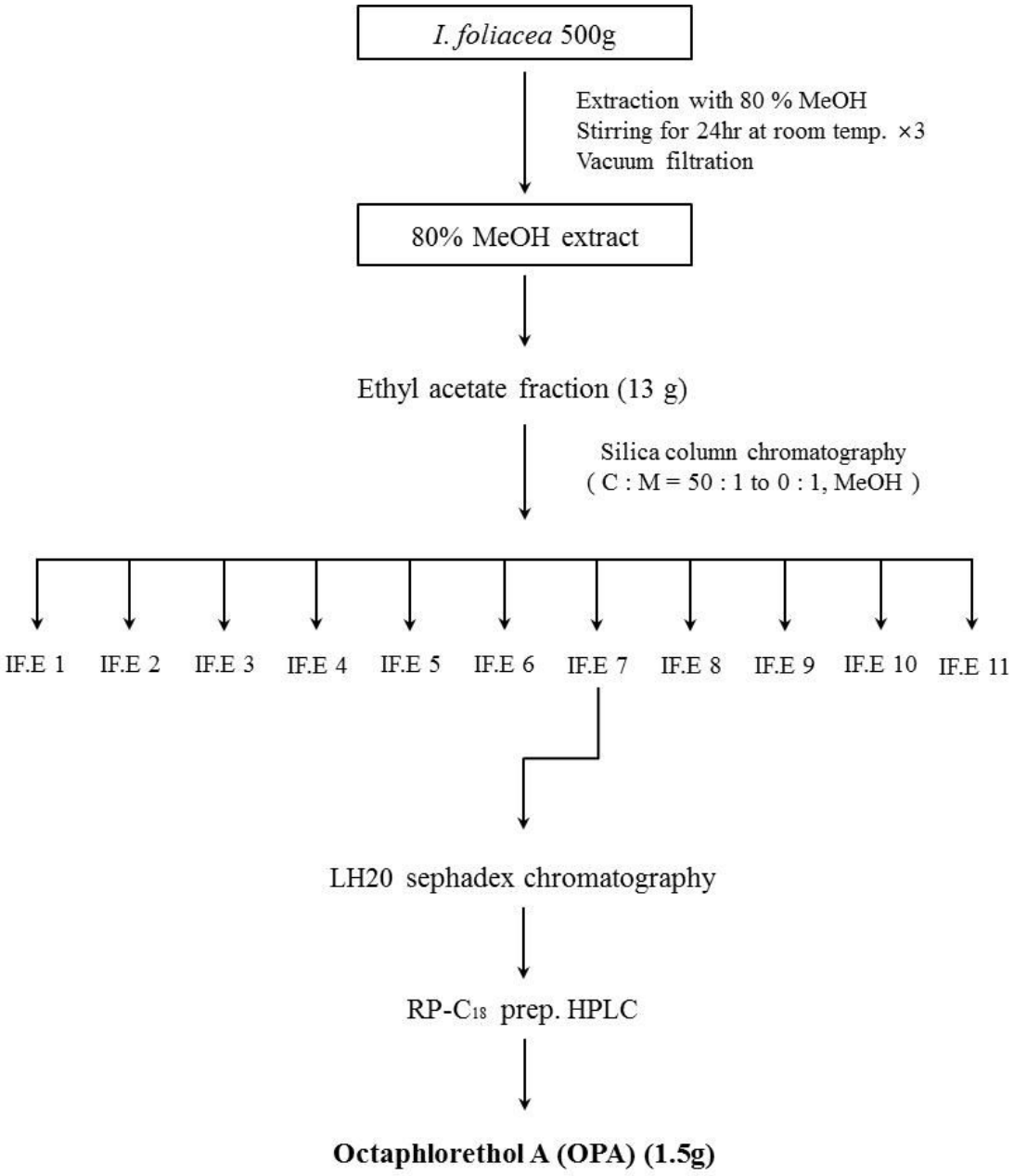

Fig. 1-6. Isolation scheme of octaphlorethol A (OPA) from *I. foliacea*

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Table 1-2. ^{1}H and ^{13}C NMR assignments for octaphlorethol A (OPA)

Position	^{13}C	^{1}H (mult. J=Hz)
1	93.9	5.58 (0.21H, d, J=2.76)
2	94.0	5.60 (0.48H, s)
3	94.1	5.68 (0.23H)
4	94.6	5.72 (0.2H)
5	94.8	5.81 (0.15H, t)
6	122.0	5.95 (0.27H, d, J=1.84)
7	123.3	6.16 (1H, d, J=1.6)
8	123.4	
9	123.5	
10	150.8	
11	151.1	
12	152.6	
13	152.7	
14	152.8	
15	152.9	
16	154.0	
17	154.1	
18	154.5	
19	156.1	
20	156.2	
21	158.6	
22	161.0	

* 400 MHz for ^{1}H and 100 MHz for ^{13}C

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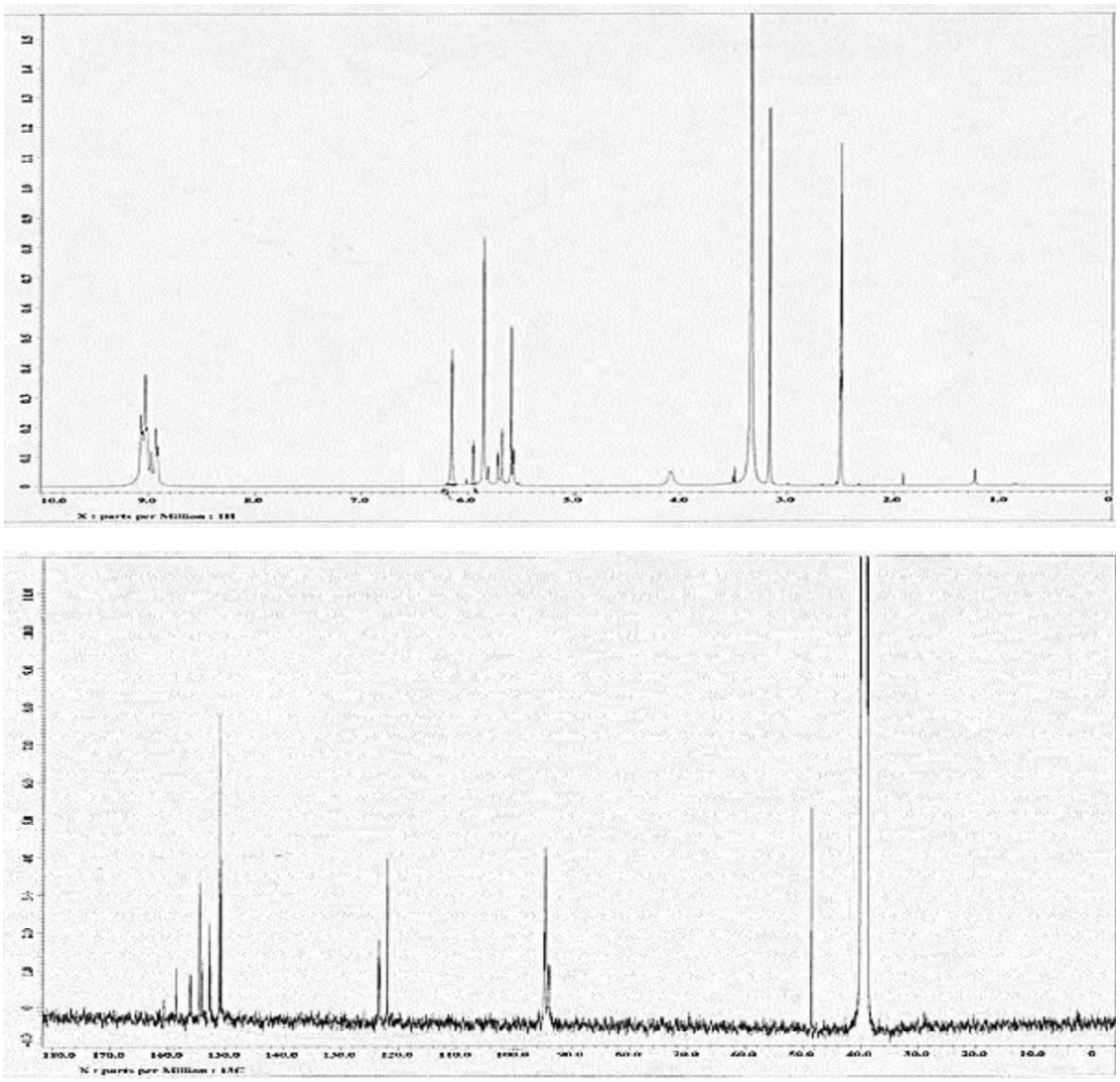

Fig. 1-7. Proton and Carbon NMR spectrum of octaphlorethol A (OPA)

(A)

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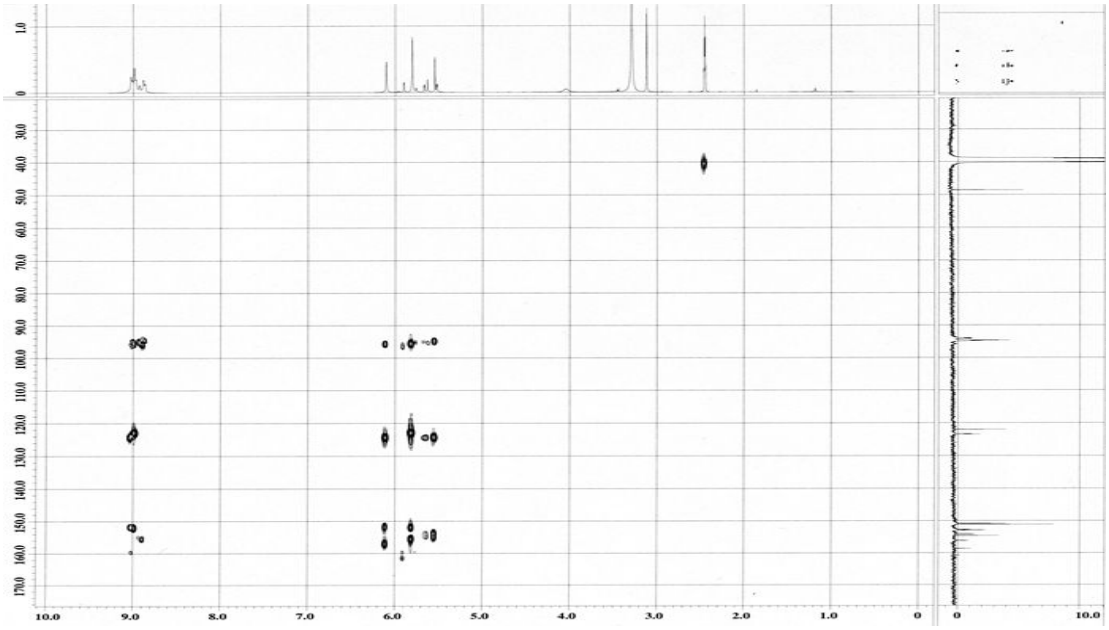

(B)

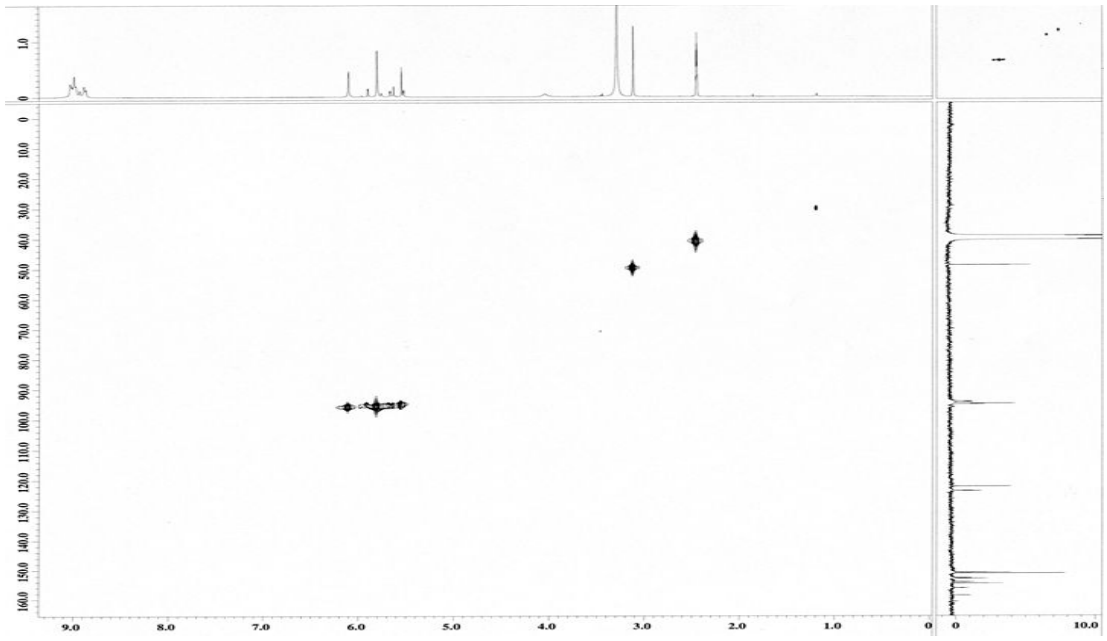

Fig. 1-8.Gradient HMBC (A) and HMQC (B) NMR spectrum of octaphlorethol A (OPA).

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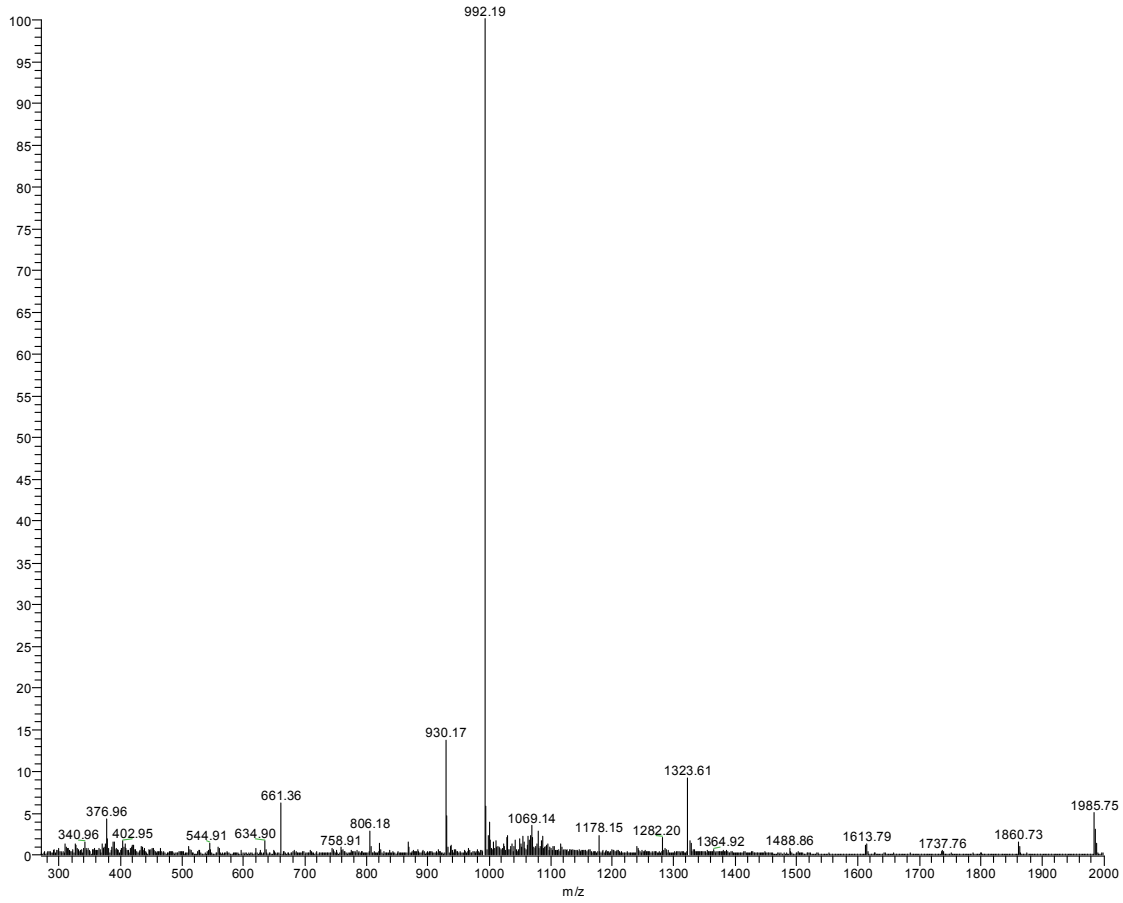

Fig. 1-9.MS spectrum of octaphlorethol A.

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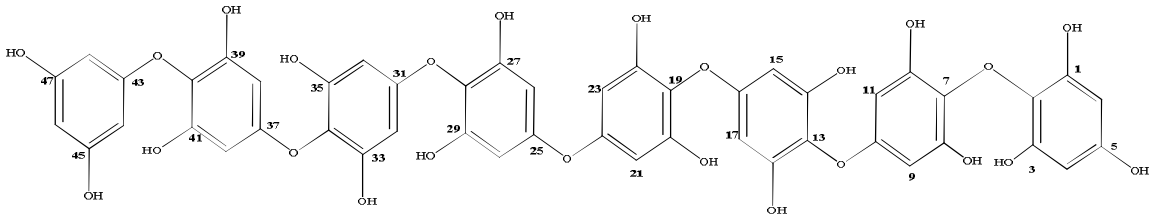

2-(4-(4-(4-(4-(4-(4-(3,5-dihydroxyphenoxy)-3,5-dihydroxyphenoxy)-3,5-dihydroxyphenoxy)-3,5-dihydroxyphenoxy)-2,6-dihydroxyphenoxy)-2,6-dihydroxyphenoxy)-2,6-dihydroxyphenoxy)benzene-1,3,5-triol

Fig. 1-10.Chemical structure of octaphlorethol A.

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Part Ⅱ.

Octaphlorethol A isolated from Ishige foliacea inhibits α-MSH-stimulated melanogenesis through MAPK and regulating tyrosinase-related protein in B16F10 melanoma cells

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ABSTRACT

One of the well-known physiological responses of human skin is melanogenesis. This is caused due to UV light, genetic reasons or other causes. We made an effort in order to look for new natural whitening agent, Octaphlorethol A (OPA). In this study, the potent skin-whitening effects of OPA isolated from *Ishige foliacea* was investigated through melanogenesis and tyrosinase inhibition activity in B16F10 melanoma cells. OPA markedly inhibits melanin synthesis and tyrosinase activity by alpha-melanocyte stimulating hormone (α-MSH) in a concentration-dependent manner. We also found that decreases microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase-related protein (TRP)-1 and -2 protein expressions.Moreover, OPA reduces p38 MAPKs protein levels and activates extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinases (JNK) protein expressions in B16F10 cells. A specific ERK inhibitor PD98059 significantly blocks OPA-inhibited melanin synthesis, whereas a JNK inhibitor SP600125 has no affect. These findings provide evidence demonstratingthat the anti-melanogenesis effect of OPA is mediated through the activation of ERK and reduces of p38 MAPKs signal pathway in B16F10 cells. Therefore, OPA would be a useful therapeutic agent for treating depigmentation and alsoan effective component in whitening cosmetics.

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Materials and methods

2.1. Materials

The marine algae were collected along the coast of Jeju island, Korea, between February and March 2010. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and the frozen samples were homogenized with a grinder prior to extraction three times.

2.2. Mushroom tyrosinase inhibition assay

Tyrosinase inhibitory activity was performed according to the method of Vanni et al., 1990. Each extract (10 μl) was diluted with 140 μl of 0.1 mM sodium phosphate buffer (pH 6.8) in a 96-well plate and then 40 μl of 1.5 mM L-tyrosine solution and 10 μl of mushroom tyrosinase (2100 units/ml) were added into a 96-well plate. The test mixture (200 μl) was mixed well and incubated at 37℃ for 12 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined by a multiplate reader at 490 nm against a blank, and the percent inhibition of tyrosinase activity was calculated using the following formula:

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

A : Absorbance at 490 nm with test sample before incubation

B : Absorbance at 490 nm with test sample after incubation

C : Absorbance at 490 nm without test sample before incubation

D : Absorbance at 490 nm without test sample after incubation

2.3. Cell culture

B16F10 mouse melanoma cells were obtained from KCLB (Korean cell line bank). The B16F10 cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (Fetal bovine serum) and 1% penicillin/streptomycin (100 X), and incubated at 37℃ under 5% CO_2 atmosphere. The cells were seeded (5 x 10^4) into 60 mm tissue culture dish, and sample treatment began 72h after seeding.

2.4. Cell viability assay

The general viability of the cultured cells was determined through the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide) to formazan(After

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were incubated at 37℃ for 24hr). After incubation, 50μl of MTT solution (stock concentration 5 mg/mL in DPBS) was added into each well and cells were incubated at 37℃ for 4 hr. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm.

2.5. Measurement of cellular melanin contents

Cellular melanin contents was measured using a previously described method (Tsuboi., 1998). B16F10 cells were treated with OPA for 3 days. Then they were washed with PBS, and were then in 1 N NaOH in 10% DMSO at 80℃ for 1hr. The relative melanin content was determined by measuring the absorbance at 475 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

2.6. Measurement of cellular tyrosinase activity

Cellular tyrosinase activity was measured according to the previously reported method with slight modifications(Tomita et al., 1992). Briefly, the cell grown in 60 mm dishes were treated as indicated for 72hr in DMEM. The cells were washed with ice-cold potassium phosphate buffered saline (PBS, pH 7.4) and disrupted in PBS containing 1% Triton X-100. Then, cells were into freezing and thawing, and the lysates

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were clarified by centrifugation at 13000× g for 10 min. After determination of protein content with a Bio-RAD protein assay kit, lysates were adjusted with lysis buffer to contain equal amounts of protein (100μg). The L-DOPA (2mg/ml) was prepared in the same lysis buffer.

Ninety microliters of each extract was placed in a 96-well plates, and the enzymatic assay was started by adding 10μl of an L-DOPA solution at 37℃. After incubation, dopachrome formation was assayed by measuring absorbance at 405nm in every 10 min for at least 1hr at 37℃ using ELISA reader.

2.7. Western blot analysis

The effect of OPA on the expression of melanogenesis-related molecules such as tyrosinase, TRP1, TRP2 and MITF were assessed by western blot analysis.

Mouse melanoma cell line B16F10 were pre-incubated for 18hr, and then stimulated with α-MSH(alpha-melanocyte stimulating hormone) in the presence of OPA for the indicated time. After 72hr incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a NucBuster™ protein extraction kit (Novagen, San Diego, CA, USA) and kept on ice for 30 min, and then centrifuged at 1600 rpm for 5min at 4℃. Cell lysates were washed by centrifugation and protein contents were

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determined with a BCA assay kit (Bio-RAD). Equal amounts of each protein extract (50 μg) were then separated on a 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels). Subsequently, proteins were electro-transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking with 5% bovine serum albumin (BSA) in TBS buffer for 2hr, the membranes were incubated with specific primary antibodies such as tyrosinase, TRP1, TRP2 and MITF. The membranes were incubated with HRP-conjugated anti-rabbit immunoglobulin (Ig) G for overnight.

The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting kit.

2.8. Statistical analysis

Date was presented as the means ±SD. All results represent three separate experiments. The results were analyzed using the ANOVA test (SPSS 14 statistical software). Significant differences between the means of parameters were determined by using the Duncan's test ($p< 0.01$).

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Results

3.1. Cell viability after exposure to OPA

We fist examined the cytotoxic effect of OPA on B16F10 cells treated with various OPA concentrations (range, 12.5-200 μM). As shown in **Fig. 2-1**. OPA did not show cytotoxic effects on B16F10 cells in the concentration range 12.5-50 μM. Therefore, to determined the effect of OPA on melanin biosynthesis and tyrosinase activity, B16F10 cells were exposed to OPA concentrations ranging from 12.5 to 50 μM.

3.2. Effect of OPA on mushroom tyrosinase activity, cellular melanin contents and cellular tyrosinase activity

The effect of OPA on mushroom tyrosinase activity and cellular tyrosinase activity are shown in **Fig 2-2** and **2-3**. OPA evidenced mushroom tyrosinase inhibitory effect of 83 % at 50 μM and the tyrosinase inhibition occurred in a dose-dependent manner. Futhermore, OPA reduced cellular tyrosinase activity in a dose-dependent manner. This results demonstrated that OPA exhibited better tyrosinase inhibitory activity than

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arbutin (100 μM), which is a direct tyrosinase inhibitor (**Fig. 2-2** and **2-3**). Moreover, 72 h treatment of OPA reduced melanin production in a dose-dependent manner compared to the arbutin. These results suggest that OPA down-regulated tyrosinase activity and that this inhibitory effect may lead to decrease cellular synthesis in B16F10 cells

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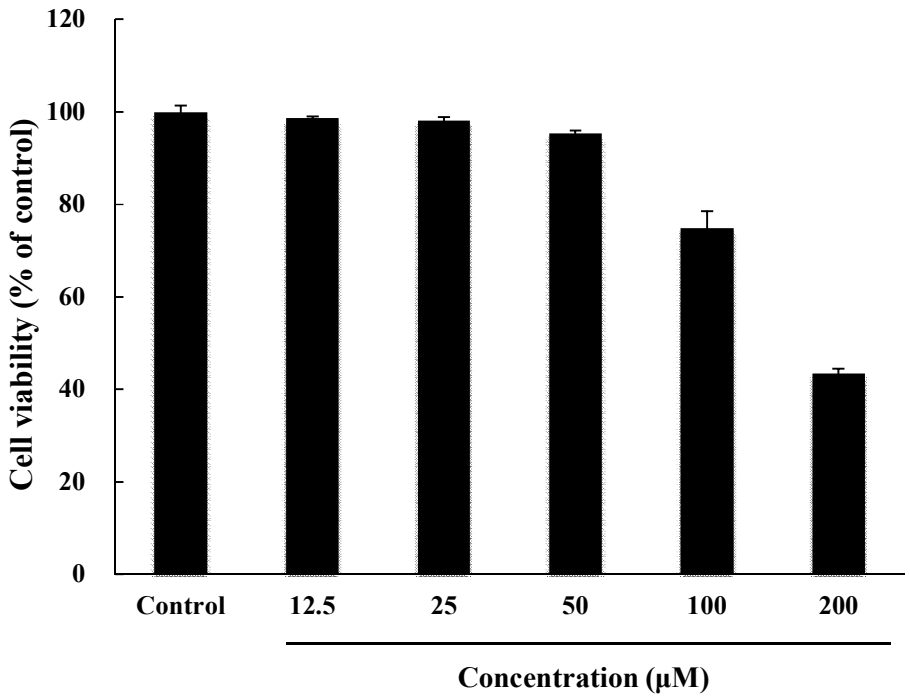

Fig. 2-1.Effect of OPA on cell viability in B16F10. Cells (1×10^5 cells/well) in wells of 96-well plates were incubated with the various concentrations of OPA for 72hr. Cell viability was determined by a MTT assay. Each percentage value in the treated cells was calculated with respect to that in the control cells.

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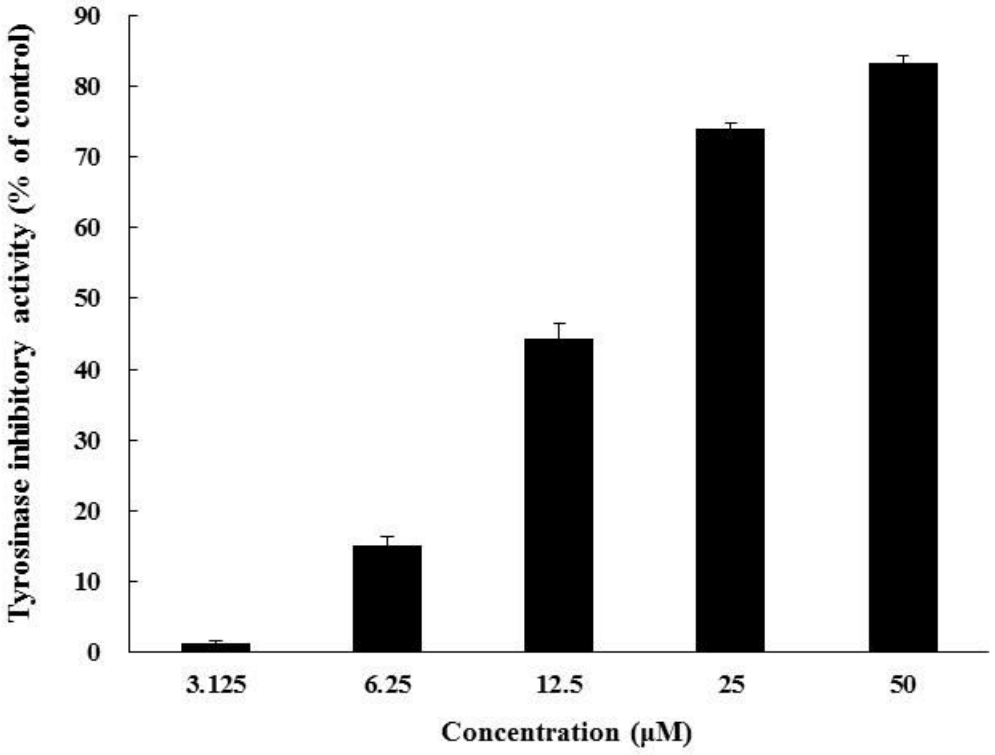

Fig. 2-2.Inhibition effect of OPA on mushroom tyrosinase activity. Assay solution contains different concentrations of OPA, 2100 units/ml mushroom tyrosinase and 1.5 mM L-tyrosine. The assay mixture was incubated 37℃ for 12min. Following incubation was determined microplate reader at 490 nm.

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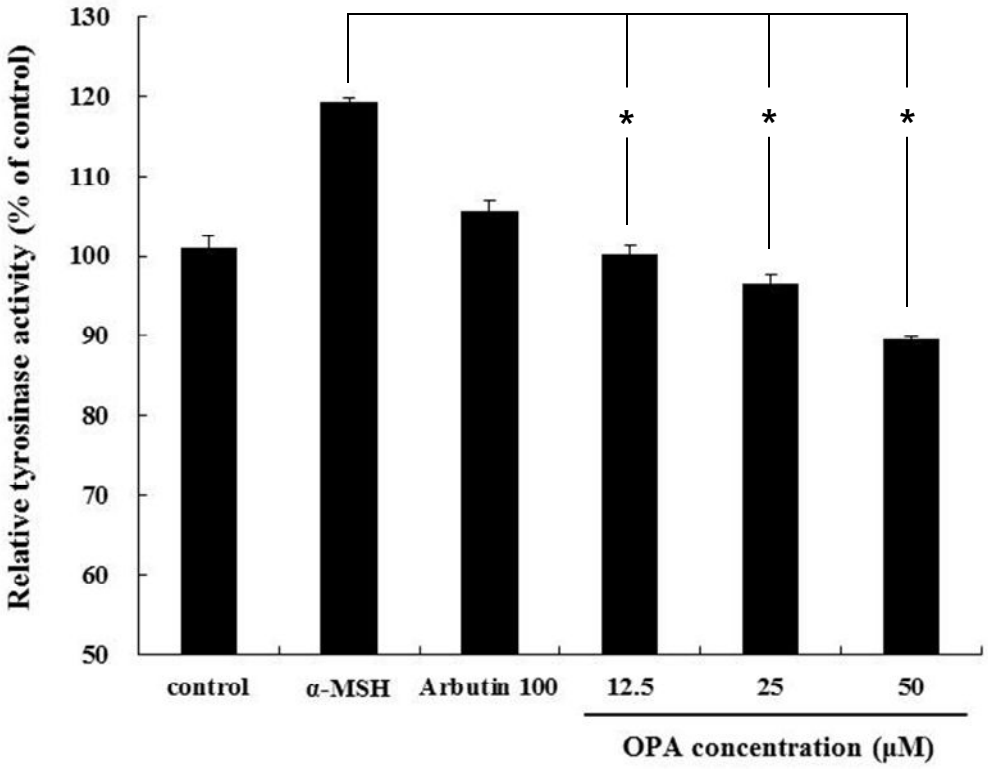

Fig. 2-3.Effect of OPA on cellular tyrosinase activity in B16F10 cells. Cells were exposed to 50 nM α-MSH in the presence of 12.5-50 μM OPA or 100 μM arbutin. The percentage values of the treated cells are expressed relatively compared to that in the control cells. Arbutin was used as a positive control for tyrosinase inhibition. Data are reported as the mean ± SEM of three independent experiments carried out in triplicate. * $p < 0.01$ compared with the α-MSH treated one.

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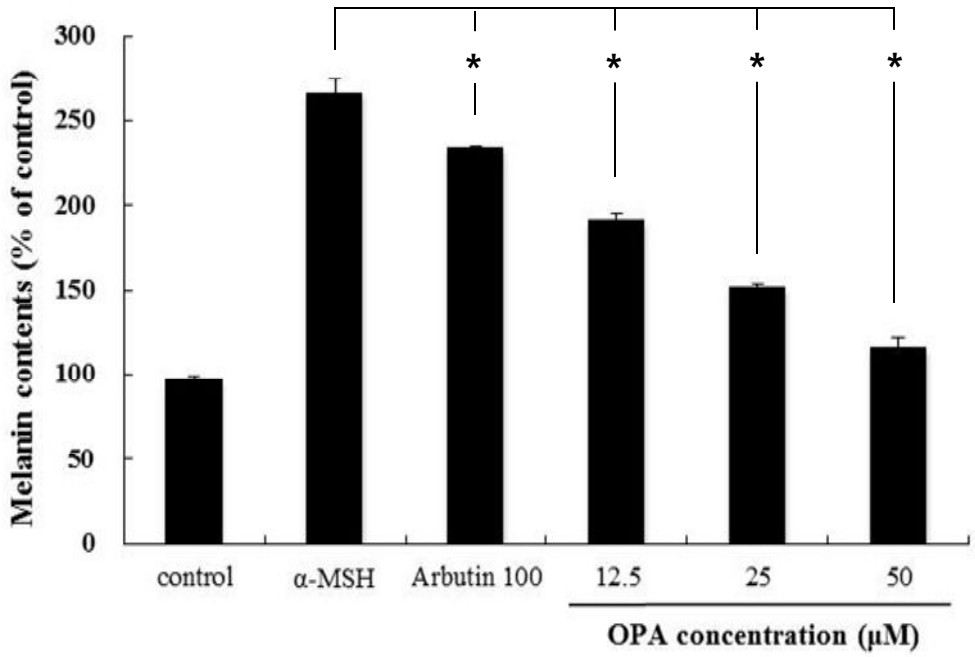

Fig. 2-4.Effect of OPA on cellular melanin synthesis in B16F10 cells. Cells were exposed to 50 nM α-MSH in the presence of 12.5-50 μM OPA or 100 μM arbutin. The percentage values of the treated cells areexpressed relatively compared to that in the control cells. Arbutin was used as a positive control for tyrosinase inhibition.Data are reported as the mean ± SEM of three independent experiments carried out in triplicate. * $p< 0.01$ compared with the α-MSH treated one.

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3.3. Effect of OPA in tyrosinase, TRP-1, TRP-2, and MITF protein expression in melanoma cells

To elucidate the mechanisms underlying the anti-tyrosinase and anti-melanogenic activities of OPA, we first examined the effects of OPA on the expression levels of the melanogenic enzymes tyrosinase, TRP-1 and -2 by immunoblotting. As shown in **Fig. 2-5**, OPA at 12.5-50 μM dose-dependently reduced the expression of the three enzymes at 72 hr.Since the three enzymes are transcriptionally regulated by MITF, we next examined the influence of OPA on MITF expression. Results showed that OPA also dose-dependently inhibited MITF expression in B16F10 cells (**Fig. 2-5**).

3.4. OPA inhibited the phosphorylation of MAPK pathway in melanoma cells

We examined the influence of OPA treatment on the activation of p38, JNK and ERK MAPKs in an attempt to further understand the molecular mechanisms involved in the hypopigmentation property of OPA by immunoblotting.As shown in **Fig. 2-6** and **-7**, ERK and JNK phosphoylation were significantly enhanced at 3hr and 12hr after OPA treatment, respectively. On the other hand, p38 phosphorylation was significantly decreased by OPA at 24 hr (**Fig. 2-8**).

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3.5. Effect of specific inhibitor on the suppressive mechanism of OPA

Since the activation of ERK and JNK seemed to decrease melanin synthesis, we examined whether the ERK and/or the JNK signaling pathways were involved in melanogenesis. Therefore, we investigated melanine synthesis and tyrosinase activity after treating with PD98059, a selective inhibitor of MEK (MAPK/ERK kinase) that is a specific upstream activator of ERK, to study the role of ERK in the OPA-induced inhibition of melanin production and tyrosinase activity. We also used SP600615, JNK inhibitor, which blocks the JNK signaling pathway. As shown in Fig. 2-9, melanin synthesis and tyrosinase activity were clearly induced after treatment with PD98059, but SP600615 has no affect (data not shown).

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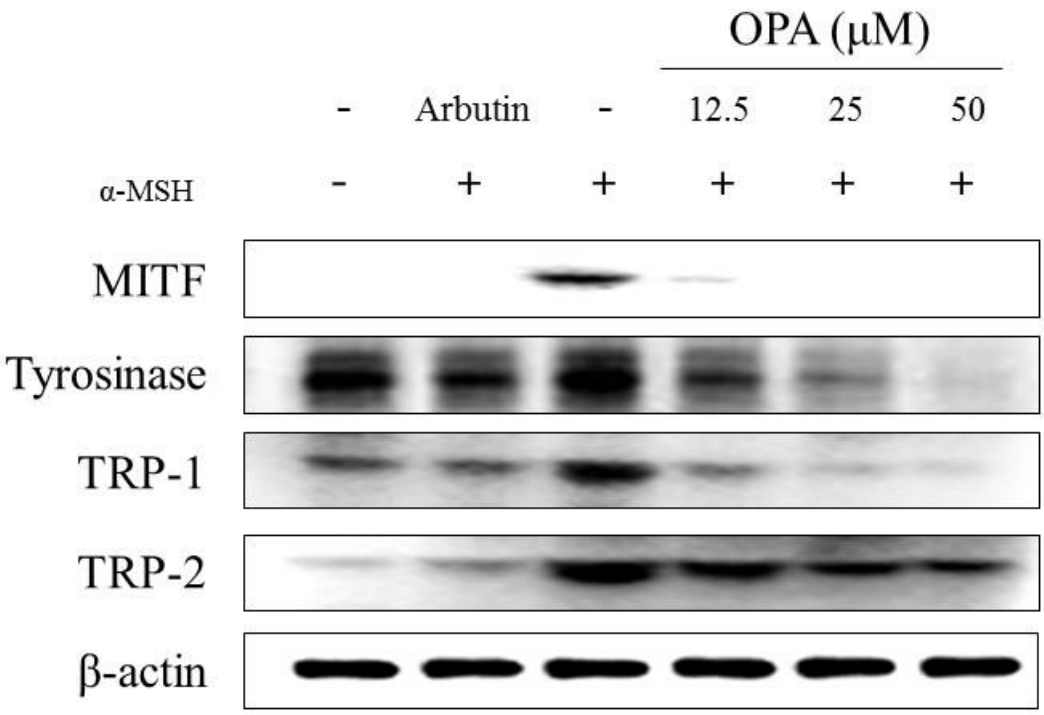

Fig. 2-5.Effect of OPA on the expression of melanogenesis-related proteins in B16F10 cells. Cells were exposed to 50 nM α-MSH in the presence of 12.5-50 μM OPA or 100 μM arbutin for 72 hr. The expression levels of the MITF, tyrosinase, TRP-1 and TRP-2 proteins were examined by western blot. Equal protein loading was confirmed by actin expression.

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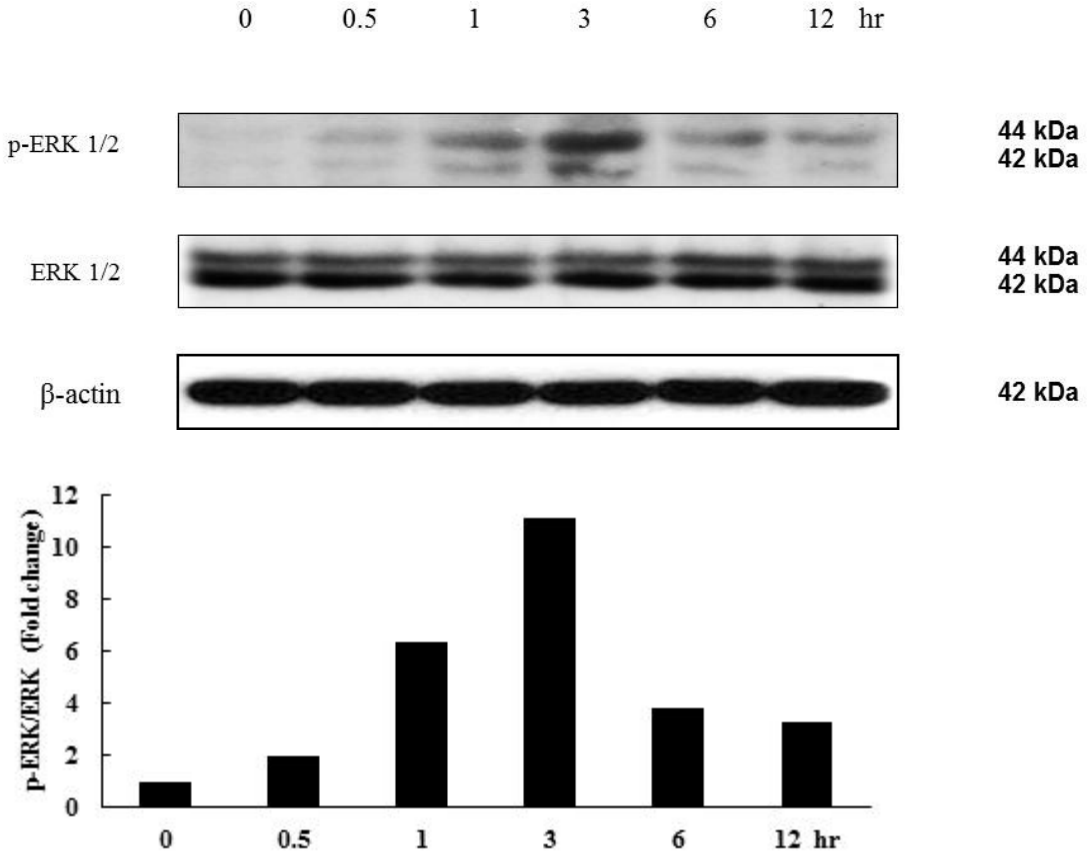

Fig. 2-6.Effect of OPA on the phosphorylation of ERK in B16F10 cells. Cells were exposed to 50 μM OPA for the indicated lengths of time following treatment. The expression levels of phospho-ERK and ERK proteins were examined by western blot. Equal protein loading was confirmed by actin expression.

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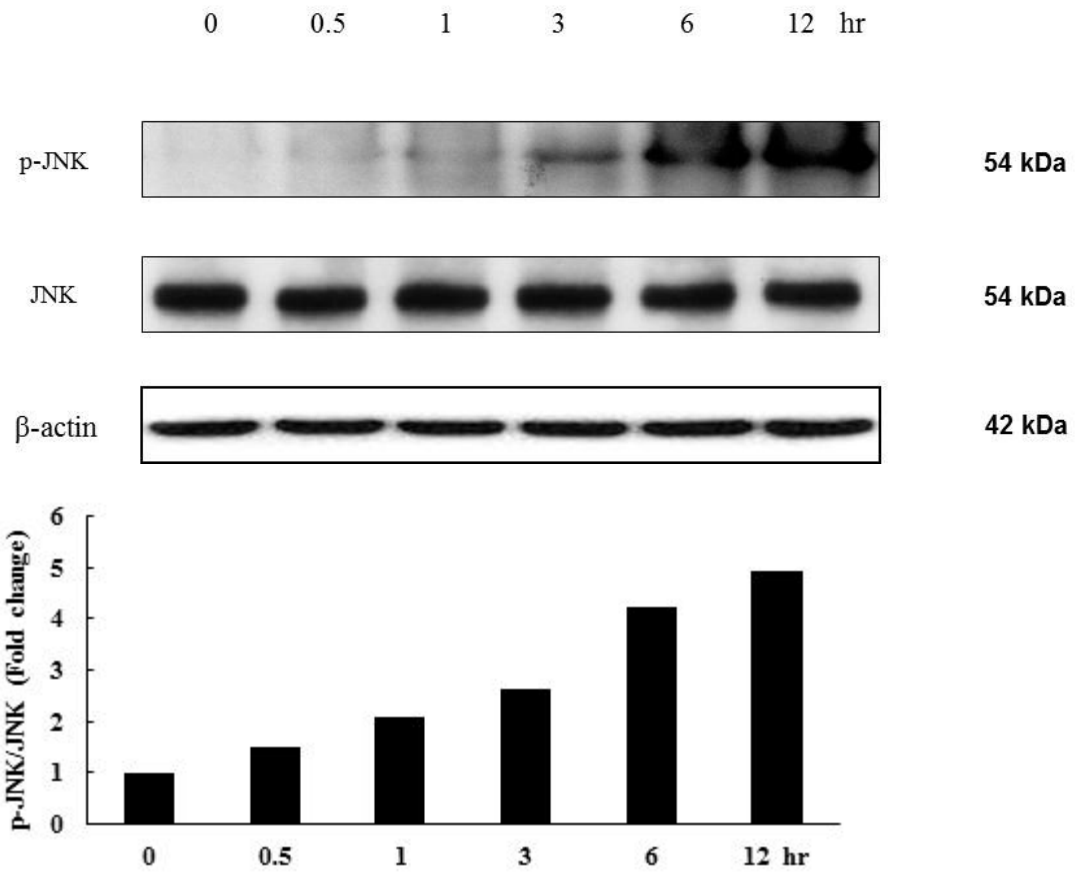

Fig. 2-7.Effect of OPA on the phosphorylation of JNK in B16F10 cells. Cells were exposed to 50 μM OPA for the indicated lengths of time following treatment. The expression levels of phospho-JNK and JNK proteins were examined by western blot. Equal protein loading was confirmed by actin expression.

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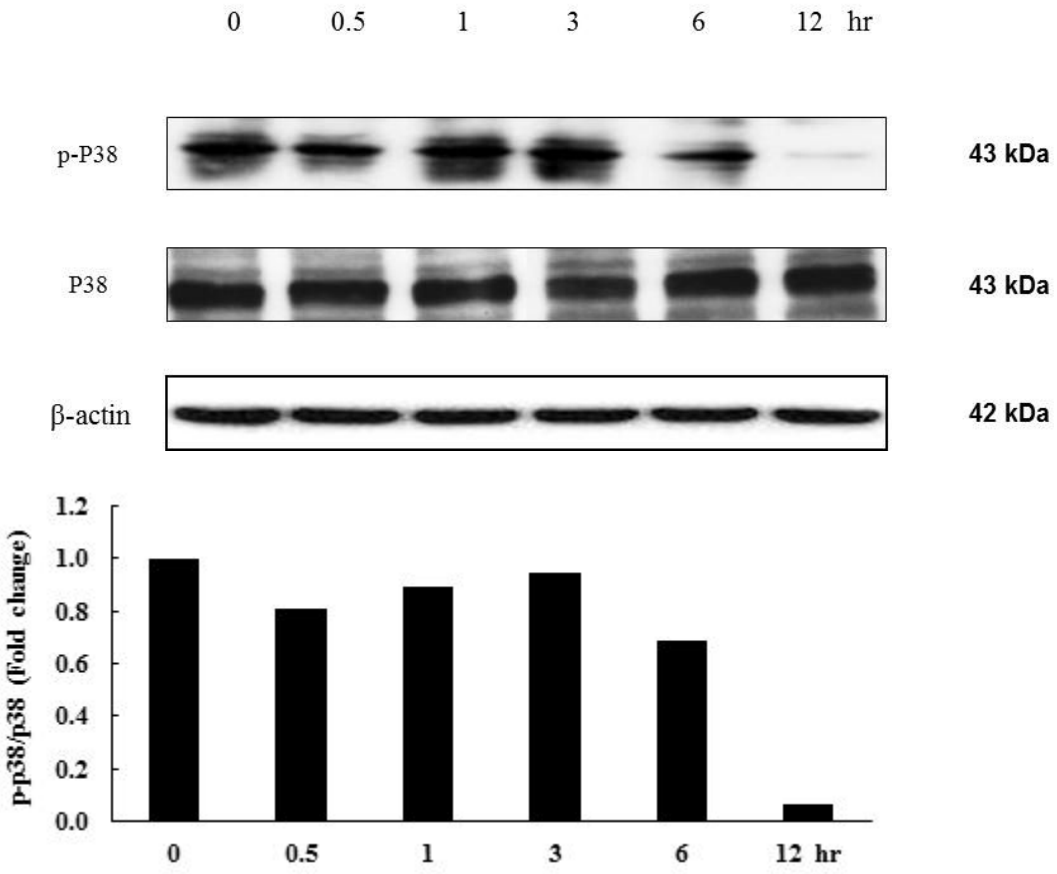

Fig. 2-8.Effect of OPA on the phosphorylation of p38 in B16F10 cells. Cells were exposed to 50 μM OPA for the indicated lengths of time following treatment. The expression levels of phospho-p38 and p38 proteins were examined by western blot. Equal protein loading was confirmed by actin expression.

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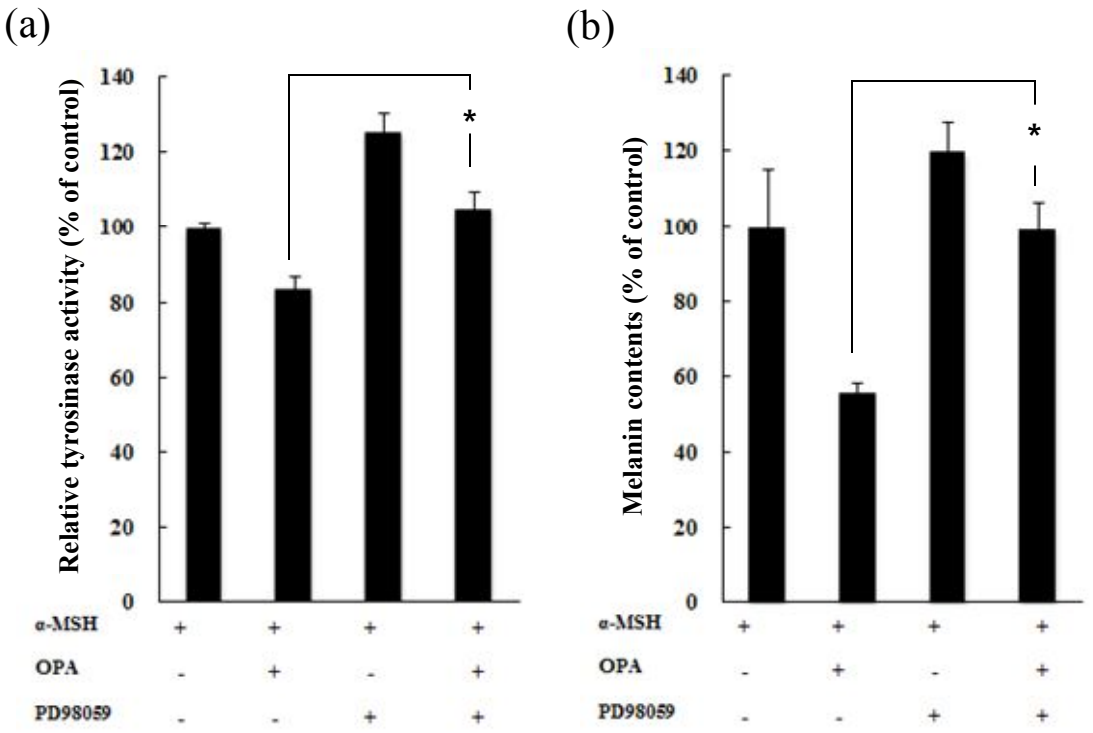

Fig. 2-9.Effect of PD98059 on cellular tyrosinase activity (a) and melanin synthesis (b) in OPA-treated B16F10 cells. Cells were exposed to 50 nM α-MSH in the presence of 50 μM OPA and 10μM PD98059. Each treated cell percentage is reported relative to that in the control cells. Data are reported as the mean ± SEM of three independent experiments carried out in triplicate. * $p < 0.01$ compared with the α-MSH and OPA co-treated one.

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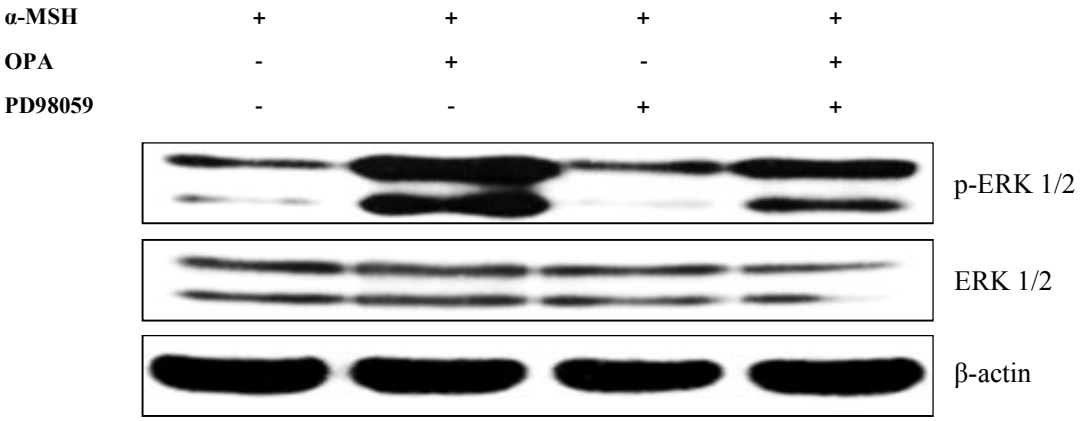

Fig. 2-10. Effect of PD98059 on the expression of the ERK phosphorylation in OPA treated B16F10 cells. Cells were exposed to 50 nM α-MSH in the presence of 50 μM OPA and 10 μM PD98059 for 48 h. The expression levels of phospho-ERK and ERK proteins were examined by western blot. Equal protein loading was confirmed by actin expression.

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Discussion

In Asian countries, many women have trouble with hyperpigmentation such as melisma and freckle. In addition, the light skin tone is an essential condition for beauty, so women want skin whitening.

The murine B16F10 cell line was used because they produce melanin, are known to contain tyrosinase which is associated with melanogenesis, respond to α-MSH activation and are easy to culture in vitro (Busca and Ballotti, 2000; An et al., 2008). Arbutin was used as a positive control in all these screenings as it has a known inhibitory effect on tyrosinase as well as melanin production.

In this study, we have firstly demonstrated the inhibitory mechanism of OPA isolated from *I. foliacea* in α-MSH-stimulated B16F10 melanoma cells. Below to 50 μM OPA did not exhibit cytotoxicity; therefore all experiments have been performed at concentration of ≤50 μM OPA.OPA exhibited better tyrosinase inhibitory activity than arbutin (100 μM), which is a direct tyrosinase inhibitor. Hence, in order to determine if OPA can inhibit melanogenesis B16F10 cells were treated with the extract before α-MSH stimulation.The melanin content of OPA-treated melanocyte; compared to the control group, treatment with OPA (12.5, 25 and 50μM) for 72 hr reduced melanin

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contents in a dose-dependent pattern. Arbutin, a positive control, also inhibited melanogenesis in B16F10 cells. On the other hand, the effect of OPA on the tyrosinase activity in cells was examined because tyrosinase is believed to play a key role in melanogenesis. Thetyrosinase activity of the OPA-treated cells was reduced to the level of the arbutin-treated cells. It had lower inhibitory activity for cellular tyrosinase.

There are three well-known enzymes, such as tyrosinase, TRP-1 and TRP-2, related to the procedure of melanin synthesis, and one transcriptional factor, MITF, regulating production of the melanogenic enzyme (). Tyrosinase inhibitory assay using L-DOPA as substrate was performed to confirm to inhibitory activity of OPA on mammalian tyrosinase activity. In the tyrosinase activity assay using intracellular system, OPA inhibited cellular tyrosinase activity. Therefore, this result suggested that OPA might be suppressed tyrosinase expression of inhibited tyrosinase activity. To confirm tyrosinase inhibitory activity was resulted from direct enzyme inhibition or reduced expression of tyrosinase, Western blot analysis was performed.The expression of proteins associated with melanogenesis B16F10 melanoma cells stimulated by α-MSH alone showed high level of tyrosinase, TRP-1 and TRP-2 in a dose-dependent manner. Furthermore, OPA inhibited the MITF protein expression in a dose-dependent manner, indicating that OPA influenced the expression of melanogenic proteins at the translational level. Inhibitory

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activity of OPA was resulted from the down-regulation of tyrosinase not by direct inhibitory activity.

To examine whether the inhibitory activity of OPA was related to the expression levels of melanogenesis-related proteins including tyrosinase, TRP-1, TRP-2 and MITF. The results show that the protein levels of tyrosinase, TRP-1, TRP-2 and MITF in α-MSH-stimulated cells were reduced by OPA treatment, consistent with OPA suppression of the expression of tyrosinase protein by down-regulating MITF expression, which is a key transcription factor of the tyrosinase gene.

The MAPK kinase family, including ERK, p38 and JNK MAPKs, plays an important role in the regulation of melanin synthesis (Smalley and Eisen, 2000; Hirata et al., 2007, Kim et al., 2007). Intracellular cAMP promotes MITF expression via the PKAdependentsignaling pathway (Bertolotto et al., 1998a,b,c). Inparticular, cAMP signaling is mediated through a PI3K-dependentmechanism via both PKA and the Ras to ERK pathways in melanogenesis(Khaled et al., 2003). Further, either elevation of cAMP levelor inhibition of PI3K expression increases melanin content by attenuationof Akt phosphorylation in human melanoma cells (Oka et al.,2000; Khaled et al., 2002). Activations of the ERK signaling (Kim et al., 2007), Akt signaling (Lee et al., 2007) and the JNK/SAPK pathways (Kim et al., 2007; Bu et al., 2008); inhibition of the p38

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pathway (Bellei et al., 2010) have been reported to be related to the down-regulation of melanogenesis. In our experiments, OPA was found to activate the ERK, Akt, JNK protein in MSH-stimulated B16F10 melanoma cells with delayed manner.

In conclusion, OPA reduced the cellular melanin content in α-MSH stimulated B16F10 cells; inhibits the activity of tyrosinase in cell and cell-free experiment; inhibits the expression of MITF, tyrosinase and TRPs in α-MSH-stimulated B16F10 cells; and phosphorylates ERK and Akt, inhibition p38 and JNK. These results suggest that OPA inhibits melanogenesis signaling by activating the p38 or Akt signal pathways-mediated suppression of MITF and its downstream signal pathway, including tyrosinase, TRP-1 and TRP-2. Therefore, this report indicates that OPA is a possible anti-melanogenic agent and might be effective against hyperpigmentation disorders or whitening cosmetics.

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PartⅢ.

Whitening effects of OPA using zebrafish *in vivo* model

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ABSTRACT

Zebrafish has been developed into an important model organism for biomedical research over the last decades. Although the main focus of zebrafish research has traditionally been on developmental biology, keeping and observing zebrafish in the lab led to the identification of diseases similar to humans. In this study, zebrafish was used as an alternative animal model to evaluate the whitening effect.

Although many hypo-pigment agents are currently available, the demand for novel whitening agents is increasing, in part due to the weak effectiveness and unwanted side effects of currently available compounds. To examine the possibility of OPA as a whitening agent, we adopted zebrafish as an alternative experiment in vivo model. OPA evidenced excellent inhibitory effects on the pigmentation of zebrafish, most likely due to their potential tyrosinase inhibitory activity. In simultaneous *in vivo* toxicity test, toxicity was observed in positive controls treated group. Moreover, OPA has been proved to possess excellent antioxidant activities (data not shown) and high phenolic content according to the previous study so that it could potentially be the candidate for cosmetic ingredients and whitening applications.

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Materials and methods

2.1. Origin and maintenance of parental fish

Adult zebrafishes were obtained from a commercial dealer (Seoul aquarium, Korea) and 10 fishes were kept in 3 *l* acrylic tank with the following conditions; 28.5℃, with a 14/10 h light/dark cycle. Zebrafishes were fed three times a day, 6 d/week, with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 min. and staged as described by Kimmel et al (1995).

2.2. Evaluation of zebrafish pigmentation

Embryos were collected and arrayed by pipette, according to the previous method (Cha et al., 2011), 15-20 embryos per well, in 24-well plates containing 475 μl embryo medium. Test compounds were dissolved in 1% dimethylsulfoxide(DMSO) 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, 0.5

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mM 1-phenyl-2-thiourea (PTU) and 20 mM arbutin were used to generate transparent zebrafish without interfering in the developmental process, and considered as a standard positive control. Phenotype-based evaluations of body pigmentation were carried out at 35 dpf. For observation, embryos were dechlorinated in 2 mg⁄ml pronase (non-specific enzyme; Sigma, St Louis, MO, USA), and photographed under a SZX9 microscope (Olympus, Tokyo, Japan).

2.3. Tyrosinase activity of OPA in zebrafish embryos

Tyrosinase inhibitory activity was spectrometrically determined as described previously (Busca et al., 1996). Briefly, approximately 100 zebrafish embryos were treated without or with melanogenic inhibitors (OPA, 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 mM $_{L}$-tyrosine was added. The control well contained 100 μl of lysis buffer and 100 μl of 1.5 mM $_{L}$-tyrosine. After incubation for 60 min at 28℃, absorbance was measured at 475 nm using the microplate reader. 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

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percentages and the results were repeated for three times in triplicate.

2.4. Melanin contents of zebrafish embryos

For the determination of melanin content, briefly, about 30 zebrafish embryos were treated without or with melanogenic inhibitors (OPA, 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℃ 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 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.

2.5. Determination of survival rate and hatching time

In order to evaluate the possible embryotoxic effects of OPA, both the concentration-dependent survival rate and the hatching time were determined (Karlsson, J et al., 2001). The numbers of hatched, hatched dead, unhatched dead, and live embryos were counted at 48 h post-fertilization (hpf). Live embryos that had not hatched at 120 hpf were counted as being alive and unhatched. Hatching time was only determined for embryos remaining alive and early mortality (prior to 48 hpf) was excluded when determining

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hatching time.

2.6. Measurement of heartbeat rate and edema size

The heartbeat rate of both atrium and ventricle wasmeasured at 35 dpf to determine the sample toxicity (Choi et al, 2007). Counting and recording of atrial and ventricularcontraction were performed for 3 min under themicroscope, and results were presented as theaverageb.p.m. Arbutin (50 mM) and PTU (0.5 mM) were used as the positive controls. Concentration of the tested sample was 100μg/ml.

2.7. Statistical analysis

Date was evaluated statically using Student's t-test or one-way ANOVA followed by Fisher's least significance was set at $p<0.001$ or $p<0.05$. The data were the mean±SD of three independent experiments.

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1. Results and Discussion

3.1. Melanin synthesis inhibitory activity of OPA in zebrafish embryo

In order to estimate the inhibitory activities, we measured the melanin synthesis using whole zebrafish extracts. We noted substantial reductions in tyrosinase activity after the treatment with OPA (Fig. 1-9). PTU and arbutin as positive controls, as anticipated, reduced melanin synthesis (44.5%, 15%) to marked degree. 3.125-25 μM OPA also inhibited tyrosinase activity (4.6%, 14.9%, 25% and 27.4%, respectively) (Fig. 3-2). Interestingly, <12.5 μM OPA have shown higher melanin synthesis inhibitory activity than arbutin. And 6.25 μM OPA has shown similar activity as arbutin. This is shown in Fig. 3-3, which contains an image of the morphologic findings. The positive controls, including PTU and arbutin evidenced a remarkable inhibition of whole body pigmentation, especially dorsal. On the other hand, OPA was utilized the melanin shrank on the surface of the trunk. In particular, The whole body pigmentation was inhibited dramatically after treatment with the OPA.

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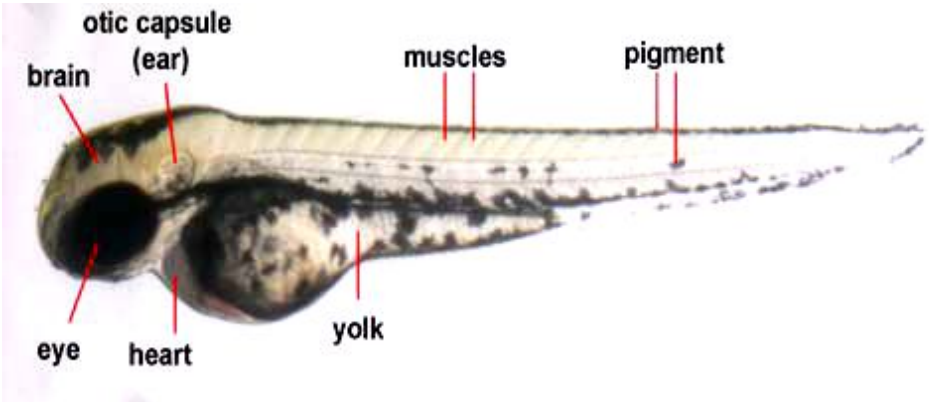

Fig. 3-1. The form of zebrafish

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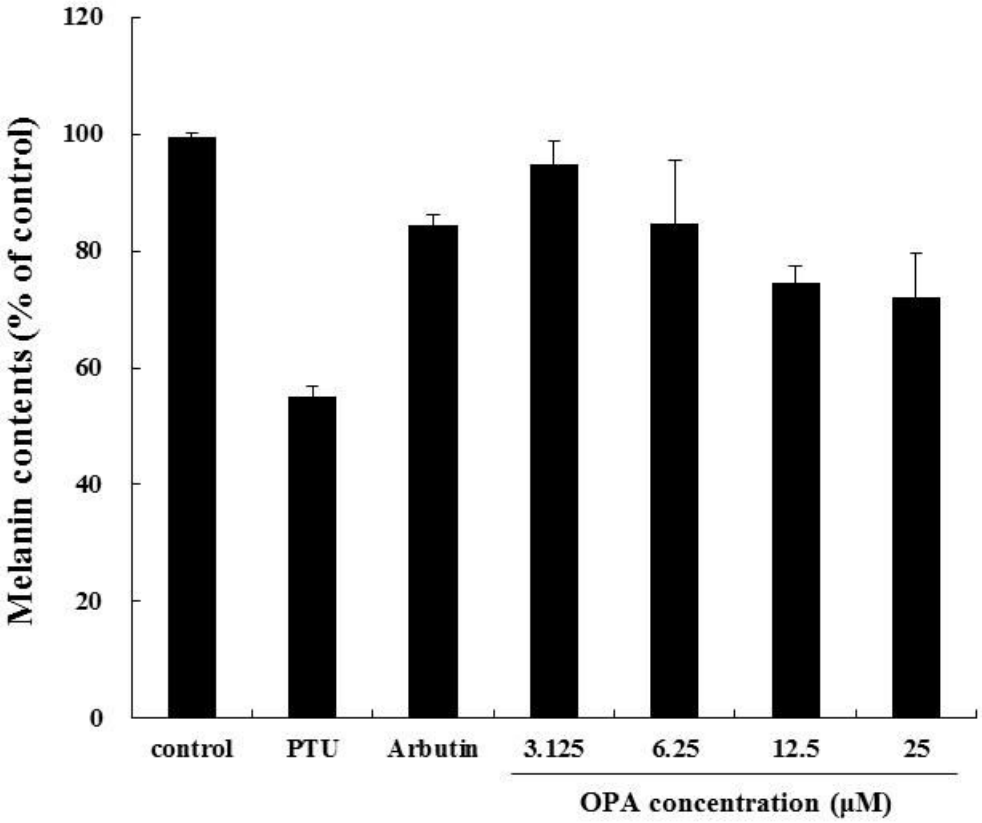

Fig. 3-2.Inhibitory effect of OPA on melanin synthesis in zebrafish. 1-phenyl-2-thiourea (PTU) and arbutin utilized as positive control. Experiments were performed in triplicate.

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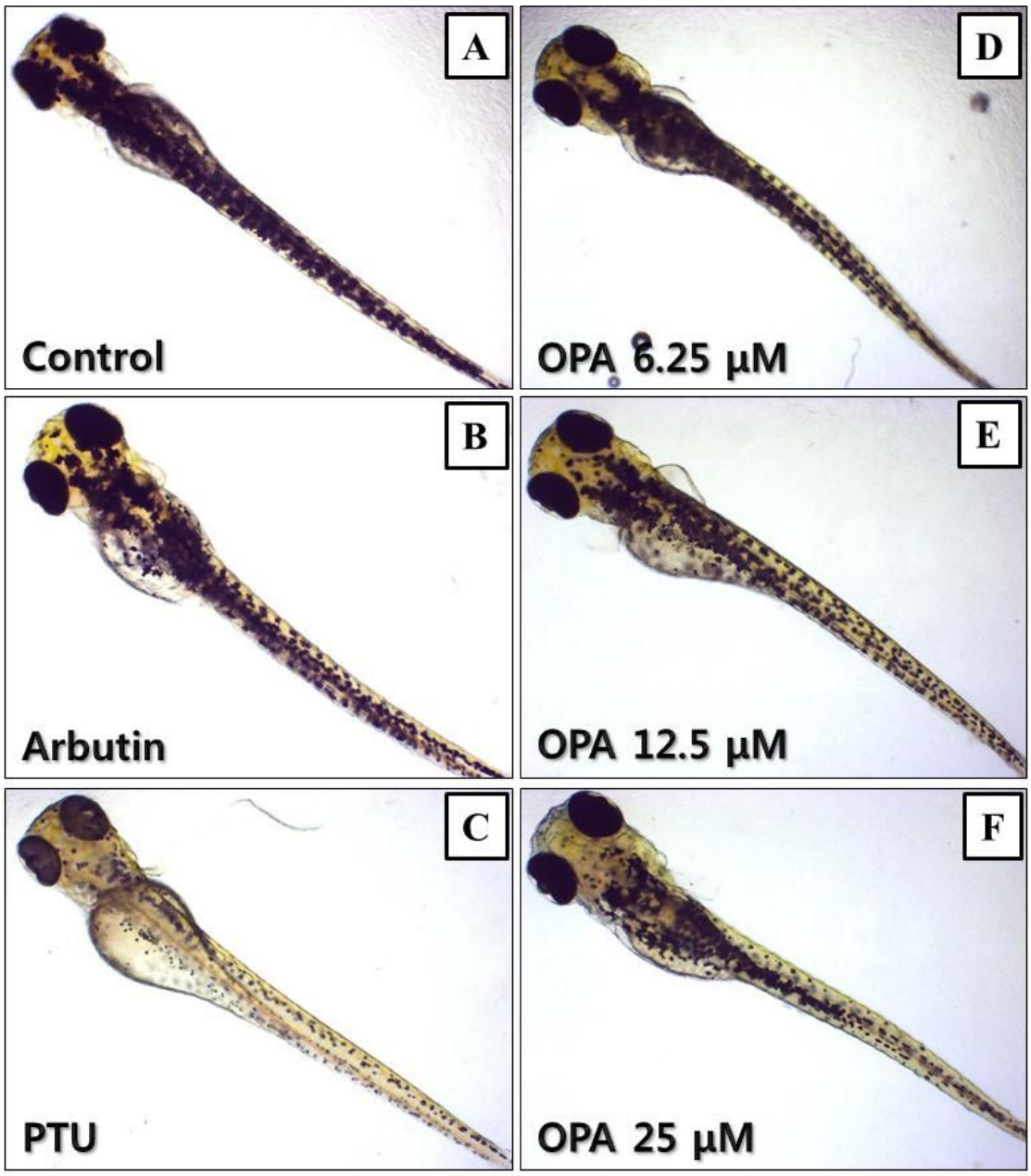

Fig. 3-3.Effect of melanogenic inhibitors on the pigmentation of zebrafish. The effect of the pigmentation of zebrafish were observed at 35 hpf (hour post-fertilization) under microscope. (A) Untreated zebrafish embryos as a control. (B, C) 1-phenyl-2-thiourea (PTU), arbutin as positive controls.(D, E, F) 3.125-25μM OPA.

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3.2. Tyrosinase inhibitory activity of OPA in zebrafish embryo

In order to estimate the inhibitory activities, we measured the tyrosinase activity using whole zebrafish extracts. We noted substantial reductions in tyrosinase activity after the treatment with OPA (Fig. 1-9). PTU and arbutin as positive controls, as anticipated, reduced tyrosinase activity (40.2%, 17.3%). 3.125-25μM OPA also inhibited tyrosinase activity (12%, 15.7%, 25.3% and 32.1%, respectively) (Fig. 3-4). >12.5μM OPA have shown higher tyrosinase inhibitory activity than arbutin. In particular, OPA increased tyrosinase inhibitory activity in a dose-dependent manner.

3.3. Toxicity of melanogenic inhibitor in zebrafish embryo

In order to determine the toxicity of the melanogenic inhibitors, we monitored the growth patterns of zebrafish. The adopted endpoints experiment used to assess the toxicity of the compounds included embryo mortality, survival rate, heart-beating and edema size disturbances. The melanogenic inhibitors (PTU, arbutin and OPA) were not associated with mortality in this experiment. On the other hand, in the heart-beat test, arbutin generated a slight disturbance, and PTU evidenced a marked increase in heart-beat rate, whereas OPA almost didn't generate any survival rate, heart-beating and

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edema sizedisturbances as compared with the controls (Fig. 3-5, 3-6, 3-7).

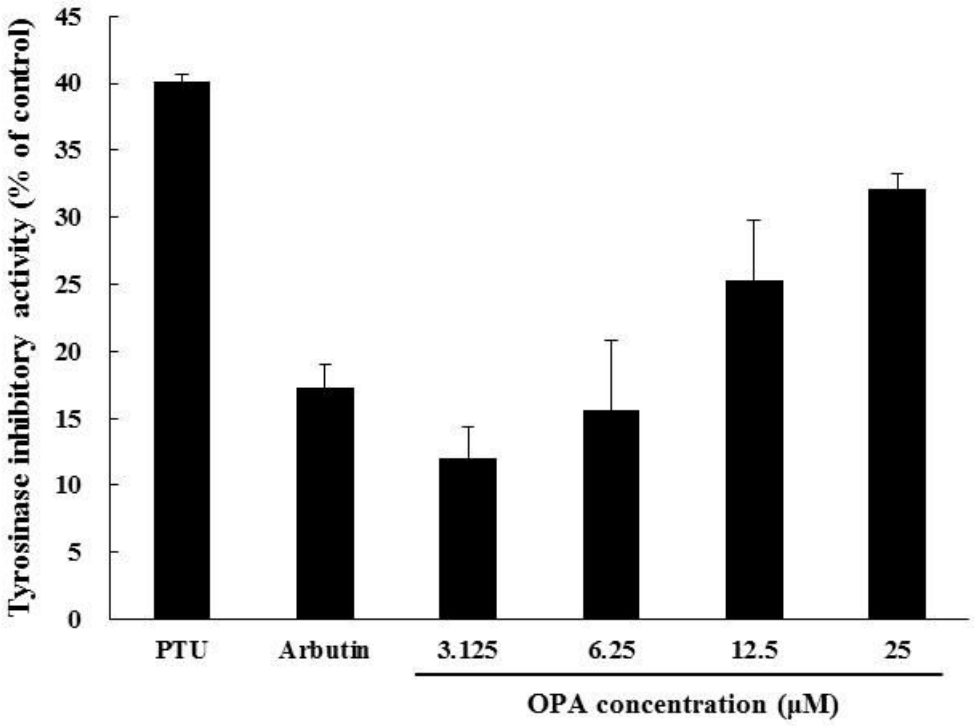

Fig. 3-4.Tyrosinase inhibitory activity of OPA in Zebrafish. 1-phenyl-2-thiourea (PTU) and arbutin utilized as positive control. Experiments were performed in triplicate.

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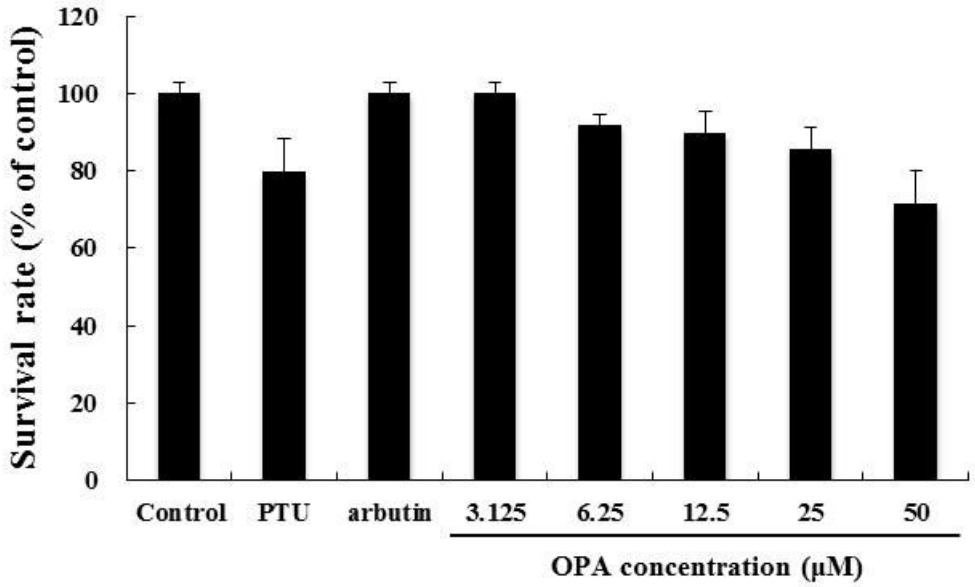

Fig. 3-5.Survival rate after treated with 50 μM 1-phenyl-2-thiourea (PTU), 500 μM arbutin and 1-50 μM OPA. Experiments were performed in triplicate.

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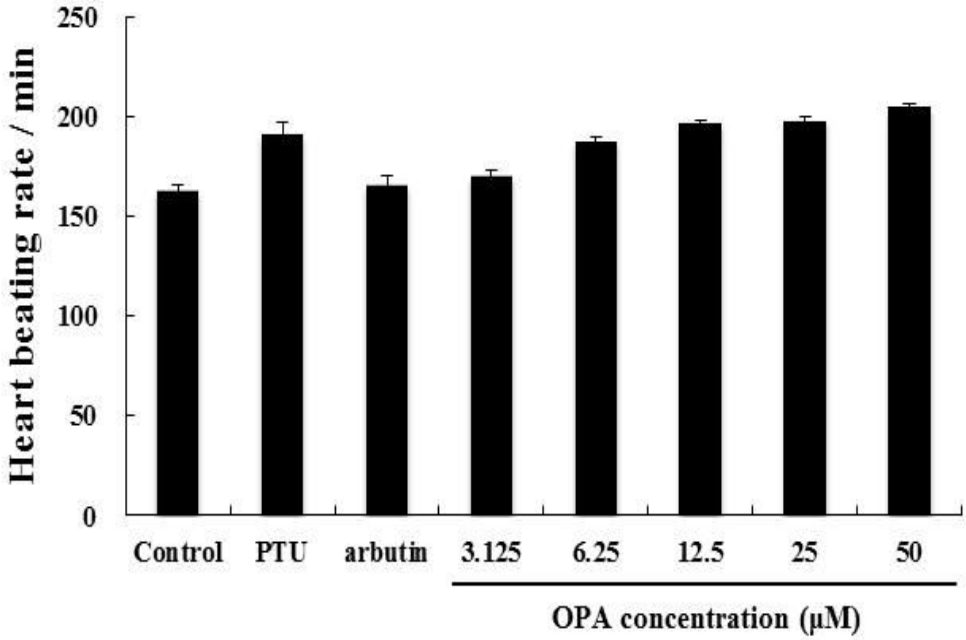

Fig. 3-6.Change of the heart-beat rate of zebrafish by OPA. The embryos were treated to 50 μM 1-phenyl-2-thiourea (PTU), 500μM arbutin and 1-50 μM OPA melanin synthesis in zebrafish. Utilized as positive controls. Experiments were performed in triplicate.

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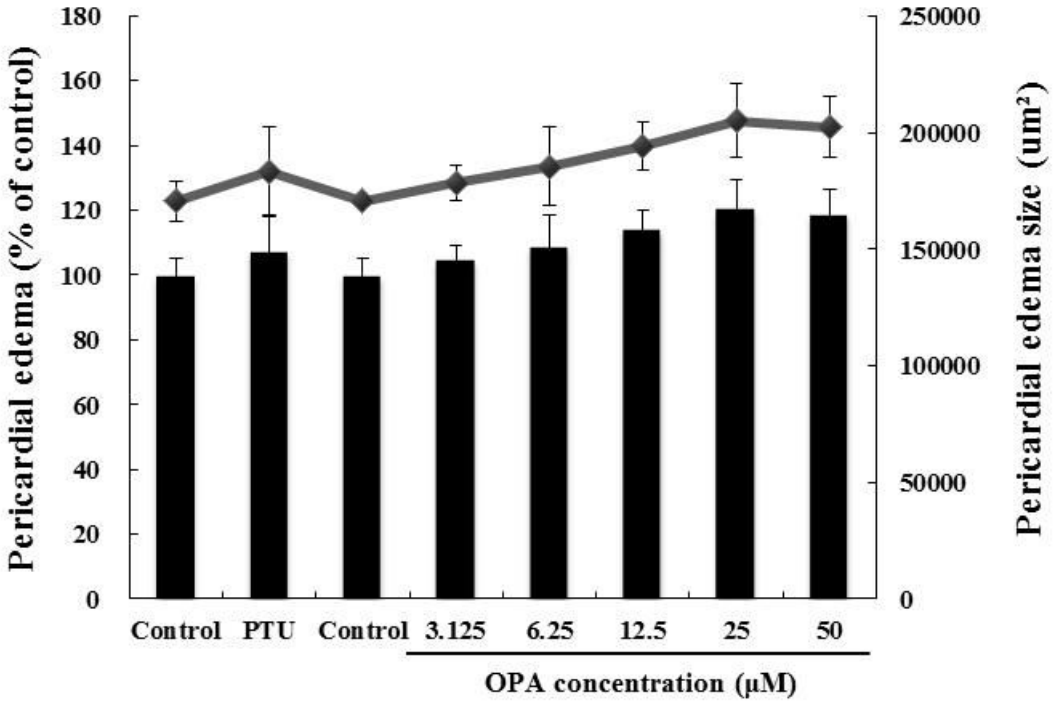

Fig. 3-7.The edema rate of zebrafish by OPA. The embryos were treated to 50 μM 1-phenyl-2-thiourea (PTU),500μM arbutin and 1-50 μM OPA melanin synthesis in zebrafish. Utilized as positive controls. Experiments were performed in triplicate.

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DISCUSSION

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. However, these tests are expensive, labor intensive and tedious, as well as it requires 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 (Zon and Peterson, 2005). Thus, we proposed zebrafish model as an in vivo animal 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 (Den Hertog, 2005; Pichler et al., 2003). Additionally, the zebrafish has melanin pigmentations on its surface, allowing for simple observation of the pigmentation process without any complicated experimental procedures. The characteristic external pigmentation pattern of the zebrafish is generated by an array of

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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) (Jin., 1999). The combimation of xanthophores and iridophores generate the yellowish-silver interstripes, whereas melanophores contribute to the formation of the characteristic longitudinal dark stripes of the epidermis. Recognizing the advantages of this model, which includes a rapid pigmentation process, permeability to small molecules and ease of handling, we propose that zebrafish can be employed as a phenotype-based model for the screening of melanogenic regulatory compounds (). Additionally the toxicity of certain compounds can be simultaneously determined by embryo mortality, survival rate, heart-beating and edema size (Zon and Peterson., 2005). In the previous studies, a variety of materials has been developed and currently utilized as cosmetic additives of as medicinal products for the treatment of hyperpigmentation (An et al., 2005; Wang et al., 2007). Recently, thedemand for natural products that inhibit or preventskin pigmentation is increasing all over the world.A variety of natural or synthetic substances are currentlyutilized as ingredients of preparations designedto control hyperpigmentation, but none of these haveproven completely satisfactory, either due to theirlimited efficacy or owing to safety concerns (Briganti et al., 2003).Forinstance,

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hydroquinone, which was used widely anduntil recently was considered the standard depigmentingagent, has now been banned for cosmeticuses in Europe and some Asian countries, and isavailable only by prescription. Kojic acid, anothertyrosinase inhibitor, has a high sensitizing potentialand has also been prohibited in some countries,citing mutagenic concerns (Petit et al., 2003). Arbutin, a natural compound,is used extensively in the cosmetic industryas a response to increasing global demand for skinwhiteningagent substances for the development ofnew depigmenting, cosmeceutical and skin lightingagents (Seo et al., 2003, Aburjai et al., 2003). In thisstudy, the cosmetic potential of OPA from *I. foliacea* was used in subsequent experiments. Therefore, we evaluated their inhibitory effects on tyrosinase and melanin synthesis in a zebrafish embryo. We evaluated the feasibility of zebrafish as an animal model system to determine the effects of OPA for melanogenic inhibition. PTU is a sulfur-containing tyrosinase inhibitor which has been extensively utilized in zebrafish research as a pigment inhibitor (Karlsson et al., 2001; Elsalini and Rohr, 2003). Arbutin was also utilized as a positive control.

Tyrosinase is an important constituent of cosmeticsand a known skin-lightening agent (An et al, 2005). Pigmentsynthesis involves the conversion of tyrosine to melaninsynthesis in melanocytes (Li et al., 2007). We utilized L-tyrosineas the substrate

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for the detection of this tyrosinaseinhibitory effect in the cells. Among the OPA, have been shown to exertpotent tyrosinase inhibitory effects (Fig. 3-4). PTU and arbutin function as whitening agentsand evidenced tyrosinase inhibitory activity.ThetestedOPA evidenced tyrosinase inhibitory activities higher than arbutin. In melanogenesis, the proximal pathwayconsists of the enzymatic oxidation of $_{L}$-tyrosine or$_{L}$-3,4-dihydroxyphenylalanine ($_{L}$-DOPA) to its correspondingo-dopaquinone catalyzed through tyrosinase (Kim et al., 2005).

In the current study, zebrafish embryos were utilized to assess melanin synthesis inhibitory activity. OPA exerted profound inhibitory effects on zebrafish pigmentation, most likely as the consequence of their tyrosinase activity inhibitory potential (Fig. 3-4, Fig 3-5). Interestingly, this ability of OPA was more profound than observed in the positive control (arbutin). Arbutin is a natural product, which it is a glycosylatedhydroquinone, and may pose similar risks of cancer (O'Donoghue, 2006). In addition, the zebrafish were allowed to continue to develop, the PTU-treated zebrafish were all dead after 4days. In simultaneous *in vivo* toxicity test, the heart-beat test, arbutin generated a slight disturbance, and PTU evidenced a marked increase in heart-beat rate, whereas OPA almost didn't generate any survival rate, heart-beating and edema size disturbances as compared with the controls(Fig. 3-5, 3-6, 3-7). Additionally

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OPA has shown in possesses excellent antioxidant activities (data not shown) and high phenolic content (Table 1-1), thus making it potential candidates for cosmetic application.

In summary, OPA from *I. foliacea* was evaluated in regard to potential efficacy as whitening agents, and they evidenced profound inhibitory effects against tyrosinase and melanin synthesis in zebrafish in vivo model. It can be surmised that these algae are likely to be useful for the cosmetic and medicinal industries.

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