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Skin whitening capability of shikimic acid pathway compounds

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Abstract. – OBJECTIVE: To examine the skin whitening capabilities of shikimic acid pathway compounds and find the most effective molecule to be used as the active ingredient for skin whitening products.

MATERIALS AND METHODS: Skin whitening is the practice of using chemical substances to lighten skin tone by the lessening the concentration of melanin. The whitening efficacy of shikimic acid pathway compounds was evaluated. Eight compounds in the shikimic acid pathway were chosen for this study: benzoic acid, p-coumaric acid, vanillic acid, syringic acid, quinic acid, shikimic acid, orcinol monohydrate, and phenyl pyruvic acid. We measured the tyrosinase inhibitory capacity of the compounds in the animal model of zebrafish and also evaluated the compounds' anti-oxidant activities using the DPPH radical scavenging, and ABTS+ free radical scavenging tests. Compounds' cytotoxicity effects were also evaluated.

RESULTS: Amongst eight shikimic acid pathway compounds used in this study, shikimic acid was the most potent tyrosinase-inhibitor and the most efficient compound to be used as an active ingredient for skin whitening. Shikimic acid revealed a good radical scavenging activity (RAS) with low cell toxicity.

CONCLUSIONS: Promising results obtained in this study may open a new window of opportunity to introduce another compound to be used in the skin-whiting cosmetics industry.

Key Words:
Tyrosinase, Zebrafish, Shikimic acid pathway, Shikimic acid.

Introduction

Nowadays, skin-caring and anti-aging cosmetics are in the center of attention in different societies in countries around the world and Asia there is a boom in this industry. Overexposure to the sun may result in some undesirable effects on the skin health. Ultraviolet ray (UV) in sunlight has a disordering effect on the sebaceous glands and damages healthy skin cells. UV stimulates tyrosinase activity in melanophores and accelerates the formation of dark spots on the skin. UV also accelerates the dermal tissue degradation and speed up the skin oxidation due to the formation of free radicals and stimulation of reactions converting dopaquinone to melanin in the melanocytes[1].

Tyrosinase inhibitors in medications and cosmetics can reduce the accumulation of melanin pigments in the skin tissue and prevent hyperpigmentation. Tyrosinase (polyphenol oxidase, EC 1.14.18.1), a copper-containing oxidase, is the rate-limiting enzyme in melanin biosynthesis. Tyrosinase catalyzes the first two steps in mammalian melanogenesis: i) transformation of the hydroxylation of tyrosine to Dopa (3, 4-dihydroxyphenylalanine) and ii) the oxidization of Dopa to Dopaquinone[2,3]. The number of studies focusing on tyrosinase inhibitory activity of various compounds is growing; this momentum is generated due to the fact that there is a substantial industrial and economic interest in these inhibitors. Our

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study was designed to study compounds that hypothetically would be good tyrosinase inhibitors.

Melanin in human skin is a major defense mechanism against UV ray. Melanin is a biopolymer molecule synthesized within epidermal melanocytes and is packaged in organelles called melanosomes. Skin color is determined primarily by this pigment. In the absence of tyrosinase activity, the melanin synthetic pathway is blocked, and the result is the formation of albino phenotype characterized by red eyes and unpigmented skin and hair[4-7]. Knowledge of melanocyte biology and the processes underlying melanin synthesis has made significant progress, creating new opportunities in the pharmacologic methods for skin darkening treatments.

The shikimic acid pathway is a metabolic pathway responsible for biosynthesis of aromatic compounds in various groups of microorganisms, plants and parasites. The shikimic acid pathway consists of seven enzymatic reactions whose end product chorismate, is the precursor for the synthesis of the aromatic amino acids such as tyrosine, phenylalanine and tryptophan. Other aromatic compounds such as coenzyme Q and vitamin K are also produced via this pathway[8,9]. The important branch point of shikimic acid pathway begins with chorismic acid itself[10-12]. Natural occurring or synthetic compounds can display inhibitory activity toward tyrosinase and effectively inhibit the formation of melanin[13-16]. These compounds may be used in skin care products as an active ingredient for skin whitening[17].

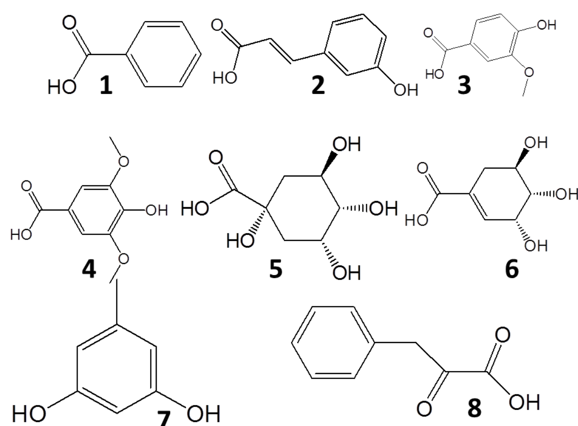

Figure 1. Eight shikimic acid biosynthesis pathway compounds employed in this study. Compounds include benzoic acid (**1**), *p*-coumaric acid (**2**), vanillic acid (**3**), syringic acid (**4**), quinic acid (**5**), shikimic acid (**6**), orcinol monohydrate (**7**), and phenylpyruvic acid (**8**).

Previous studies[13,14,16] showed that some phenolics compounds, such as caffeic acid and ferulic acid were tyrosinase inhibitors. Eight compounds in the shikimic acid pathway were chosen for this study: benzoic acid, *p*-coumaric acid, vanillic acid, syringic acid, quinic acid, shikimic acid, orcinol monohydrate, and phenyl pyruvic acid (Figure 1). We used zebra fish as our animal model to verify the whitening capability of synthetic compounds in the shikimic acid pathway. Zebrafish have similar developmental pattern and gene homologies to those of mammals; therefore zebra fish are often used in drug development projects[7,11]. Zebrafish is an alternative *in vivo* model to determine the effects of melanogenic inhibition.

The security issue was crucial for this study and we analyzed the survival rates of murine melanoma B16 cells and human skin fibroblast Hs68 cells to ensure the safety of the compounds to be potentially used in skin-care products[18,19].

Materials and Methods

Chemical Teagents

L-ascorbic acid (AA), *p*-coumaric acid, orcinol monohydrate, 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\cdot+}$), 4-Hydroxyphenylâ-D-glucopyranoside (arbutin), mushroom tyrosinase (EC.1.14.18.1), 3,4-dihydroxy-L-phenylalanine (L-DOPA), potassium phosphate dibasic (KH_2PO_4), ferric chloride ($FeCl_2$) and potassium dihydrogen phosphate (KDP), were purchased form Sigma Chemical Co. (St. Louis, MO, USA). Arbutin and ascorbic acid were dissolved at a concentration of 10 mM in dimethyl sulfoxide (DMSO) stock solution. Benzoic acid, gallic acid, vanillic acid, syringic acid, quinic acid and shikimic acid were purchased from Alfa Aesar Co., Taiwan. All reagents used in this study were analytical grade. The water used was re-distilled and was ion-free.

Cell Culture Human Skin Fibroblast

Hs68 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Human premalignant keratinocytic HaCaT cells were kindly provided by Prof. Hamm-Ming Sheu (National Cheng Kung University Medical College, Tainan, Taiwan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA) that was supplemented with 10% fetal bovine serum (Ha-

zelton Product, Denver, PA, USA) and 1% penicillin-streptomycin at 37°C in 5% CO_2.

Zebrafish Whitening Drug Screen Test

The eight compounds from shikimate pathway were tested for their melanogenic inhibition capability on the pigmentation of zebrafish embryos. Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Seoul, South Korea) and ten fish were kept in a 3-L acrylic tank at 28.5°C, with 14:10 hours light: dark cycle. Zebrafish were fed three times a day, 6 days a week, with Tetramin flake food supplemented with live brine shrimps *(Artemia salina)*. Embryos were obtained from natural spawning that was induced in the morning by turning on the light. A collection of embryos was completed within 30 mins[18]. Synchronized embryos were collected and arrayed by a pipette. We prepared 24-well plates containing 475 µL embryo medium with 10 to 15 embryos per well. Test compounds were dissolved in 1% dimethylsulfoxide and then added to the embryo medium from 9 to 35 hours 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 (used as standard positive control). We used the Image J software which is a set of Image processing software, developed by U.S. National Institutes of Health. Currently, this software is widely used for image processing purposes in various studies. Data were expressed as mean percentages, and the experiments were conducted in triplicates[19].

Cellular Tyrosinase Activity Assay in B16 Cells

Cellular tyrosinase activity assay in B16 cells was studied by measuring the oxidation rate of DOPA. One milliliter aliquots of cells, with a concentration of 10^5 cells per mL, were plated in 24-well multi-dishes and incubated for at least 24h prior to use. They were washed with 1X PBS and lysed in 900 µL of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and then subjected to the freeze-thaw cycle. They were kept at −80°C for 30 min and transferred to 25°C and kept at this temperature for 25 min and then in 37°C for 5 min. After that, we added 100 µL of 10 mM DOPA to them. Following incubation at 37°C for 4 h, the absorbance of the agents was measured at 475 nm (Synergy2, BioTek Instruments, Inc., Winooski, VT, USA). The data were expressed as mean percentages and the experiments were conducted in triplicate[17].

Radical cation ABTS•+ (2,2′–Azino-di[3-ethylbenzthiazoline sulfonate]) Scavenging Activity

For DPPH· radical scavenging activity analysis we added various compounds from shikimic acid biosynthesis pathway, in equal volumes, to the methanol solution with different concentrations (1, 2, 4, 8, and 16 µM). After incubation at 25C for 30 min in dark, the absorbance (A) was measured at 517 nm (Hitachi U-2001, Japan). For ABTS•+ radical scavenging activity analysis, we dissolved ABTS•+ in water to 7 mM. ABTS•+ radicals were produced by reacting ABTS•+ stock solution and 2.45 mM potassium persulfate. Mixture was kept in the dark at room temperature for 12–16 h. The ABTS•+ radical solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm at 30C. Each agent reacted with 2.9 ml of diluted ABTS•+ radical solution for 20 min at 30°C (100 µg/ml), and then the absorbance was measured at 734 nm (Hitachi U-2001, Tokyo, Japan). Ethanol or distilled water was used as negative controls. L-ascorbic acid (AA) was used as a positive control. The scavenging ability of compounds or AA in DPPH· and ABTS•+ was calculated using the following equation:

$$= \frac{\text{Radical scavenging ability (\%)}}{(1 - \text{Sample/Control})} \times 100$$

Statistical Analysis

Data were calculated as Mean ± SD. Statistical analyses were carried out by analysis of variance (ANOVA) followed by appropriate posttests including multiple comparison tests (LSD). All analyses were done with SPSS statistical software package and a probability value of less than 0.05 was considered as statistically significant.

Results

Zebrafish Whitening Drug Screen Test

The effects on the pigmentations of zebrafish embryos were observed under the microscope. The areas with color pigmentation in the control were considered to represent 100%. Benzoic acid (**1**), *p*-coumaric acid (**2**), vanillic acid (**3**), syringic acid (**4**), quinic acid (**5**), and shikimic acid (**6**), in

Table I. Tyrosine inhibition effects of eight compounds on B16 cells.

Compounds	Conc. (μM)	24 H	72 H
		Inhibition (%)	
Control	0	0.0 ± 0.4[a]	00.0 ± 8.2
Phenylpyruvic acid (8)	100	24.9 ± 7.5	35.7 ± 2.6
Benzoic acid (1)	200	3.9 ± 0.7	67.2 ± 8.3
P-coumaric acid (2)		2.2 ± 0.7	00.0 ± 7.4
Quinic acid (5)		2.5 ± 0.7	23.5 ± 6.2
Orcinol monohydrate (7)		2.0 ± 0.7	76.6 ± 2.9
Vanillic acid (3)	500	1.4 ± 1.6	37.7 ± 2.3
Syringic acid (4)		3.4± 2.0	1.9 ± 6.7
Shikimic acid (6)		3.1 ± 0.4	85.4 ± 1.9

100 μM or 200 μM concentrations revealed some melanogenic inhibition activities (Figure 2).

These compounds were able to reduce pigmentation area in zebrafish embryos when compared to arbutin. In higher concentration (500 μM), shikimic acid demonstrated more intense inhibitory effects on zebrafish pigmentation due to its tyrosinase-inhibitory capacity (inhibition reached to 78.1 ± 5.0 %) (Figure 3).

Zebrafish system is a new alternative to mammalian models, due to the fact that melanin is found on the body surface of the zebrafish. This system has several advantages including the rapidity, cost-effectiveness, and physiological relevance. Presence of the melanin pigmentations on the surface of the zebrafish makes the fish suitable for simple observation of the pigmentation process without the need for any complex experimental procedures.

Tyrosinase Inhibition Effect of Eight Compounds on B16 cells

We examined the tyrosinase inhibition activities of eight compounds of Shikimic acid on B16 cells for 72 hrs. When 8 compounds were tested at 100 μM, phenyl pyruvic acid showed a weak tyrosinase-inhibitor activity. At 200 μM, benzoic acid and orcinol monohydrate exhibited strong inhibition activity with 67.2 ± 8.3 % and 76.6 ± 2.9 % at 72 hrs, respectively, At 500 μM, shikimic acid showed significant tyrosinase inhibition activity with 85.4 ± 1.9 % at 72 hrs. At this concentration, other compounds did not have any significant tyrosinase inhibition activity (Table I).

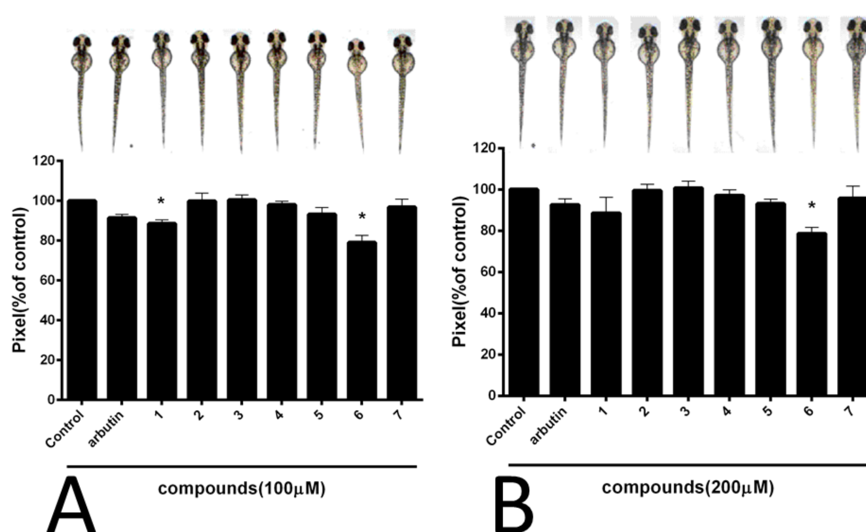

Figure 2. Effects of eight shikimic acid pathway compounds on pigmentation of zebrafish at 100 mM *(A)*, 200 mM *(B)*. Arbutin as control group, and phenylpyruvic acid (8) had no effects on pigmentation.

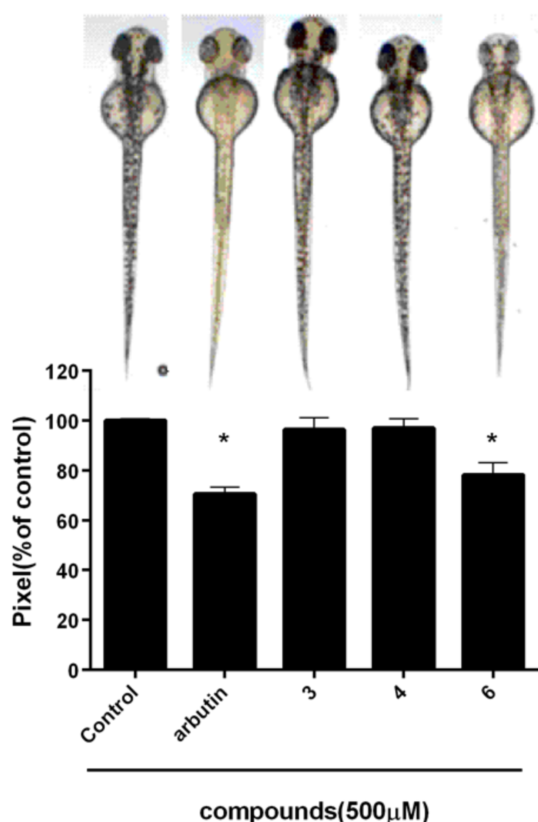

Figure 3. Effects of eight shikimic acid pathway compounds on pigmentation of zebrafish at 500 mM. Arbutin was used as control group. The compounds **1**, **2**, **5**, **7** and **8** had no effects.

The Cytotoxicity test on Hs68 and B16 Cell

The security issue is crucial for the development of whitening materials. Survival rate and cell viability of murine melanoma B16 cells and human skin fibroblast Hs68 cells were examined using an MTT assay. The results of toxicity test of shikimic acid pathway compounds on the Hs68 cells with 100, 200 and 500 μM concentrations for 24 hrs revealed that at 100 μM phenyl pyruvic acid had medium cytotoxicity and at 200 μM benzoic acid exhibited slight cytotoxicity. Viability of the Hs68 cells was 78.7 ± 5.3%. The rest of the compounds had no significant cytotoxicity at any concentration (Table II).

Toxicity test (72 hrs) on B16 cells at 100, 200 and 500 μM concentrations revealed that at 100 μM phenyl pyruvic acid had medium cytotoxicity, and at 200 μM benzoic acid and *p*-coumaric acid showed slight cytotoxicity to 59.9 ± 3.5% and 76.7 ± 1.3%, respectively. At 500 μM syringic acid exhibited medium cytotoxicity (viability of B16 cells was 49.7 ± 5.0%). Moreover, shikimic acid exhibited no significant cytotoxicity at any concentration used in this study (Table II)

Radical cation ABTS▪+(2,2-Azino-di[3-ethylbenzthiazoline sulfonate]) Scavenging activity

Previous studies demonstrated that once the tyrosinase activity in B16 cells was inhibited using an active compound, the compound also exhibited free radical scavenging activity[12]. Because shikimic acid exhibited an inhibiting effect on tyrosinase, we chose it for further experimental tests. DPPH free radical scavenging of shikimic acid was IC_{50} at a concentration of 3.06 ± 0.05 μM. ABTS•+ free radical scavenging test showed that shikimic acid had IC_{50} free radical scavenging in 3.99 ± 0.02 μM (Figure 4).

Discussion

In this study, we used zebrafish which has a high gene homology to mammals as well as a de-

Table II. Cytotoxicity tests on Hs68 and B16 cell.

Compounds	Conc. (μM)	Hs68 24 H Viability (%)	B16 72 H Viability (%)
Control	0	100.0 ± 4.3[a]	100.0 ± 0.9
Phenylpyruvic acid (8)	100	066.5 ± 9.1	54.9 ± 7.2
Benzoic acid (1)	200	078.7 ± 5.3	059.9 ± 3.5
P-coumaric acid (2)		099.5 ± 3.1	076.7 ± 1.3
Quinic acid (5)		094.0 ± 3.9	095.3 ± 4.5
Orcinol monohydrate (7)		101.7 ± 0.6	104.4 ± 2.6
Vanillic acid (3)	500	108.4 ± 1.5	100.3 ± 1.3
Syringic acid (4)		094.1 ± 2.8	049.7 ± 5.0
Shikimic acid (6)		098.8 ± 2.6	100.6 ± 0.5

[a] Values are represented as mean ± standard deviation (n=3)

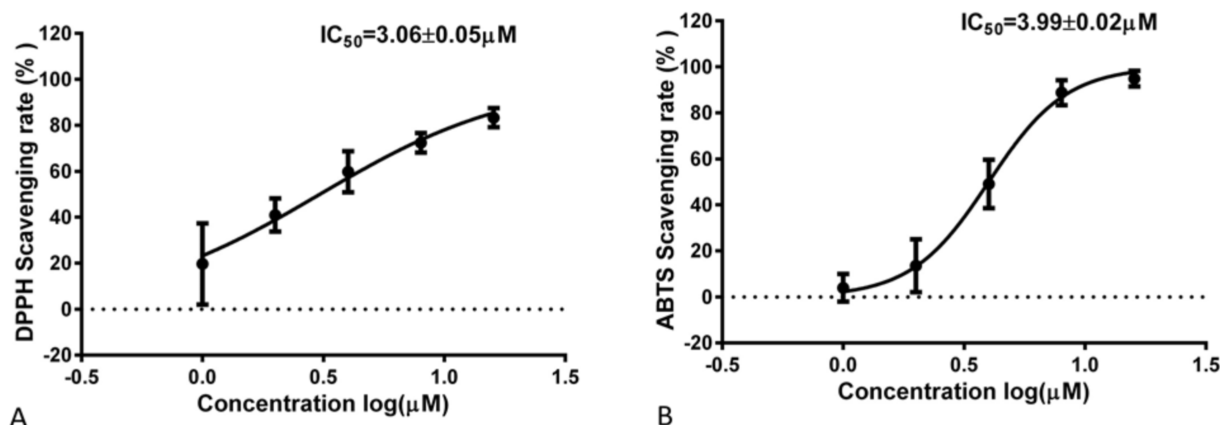

Figure 4. Free radical scavenging activities: *(A)* DPPH• and *(B)* ABTS•+ radical scavenging capacities. Each value represents the mean ± SD from triplicate experiments.

velopmental pattern close to these animals. Zebrafish is often used in drug development studies[13]. Zebrafish whitening evaluation experiments were conducted to verify the whitening and anti-oxidation capacities of synthetic compounds in shikimic acid circulation, in order to find more ingredients which can be used for the whitening and anti-oxidation purposes.

The results of melanin-inhibition assay of embryo zebrafish demonstrated that the total area of melanin had decreased to 79.1 ± 3.6% after treating with shikimic acid (6) which was a more pronounced drop when compared to the control group. Results obtained from the tests performed on B16 melanoma cells, to evaluate the levels of tyrosinase activity inhibition, showed that while inhibitory effects of benzoic acid (1), shikimic acid (6) and orcinol monohydrate (7) were over 50%, shikimic acid (6) demonstrated the most significant inhibitory effect (76.6 ± 2.9%). The effects of shikimic acid biosynthesis compounds on the melanin levels of zebrafish embryos were examined and the results showed that shikimic acid (6) was the most effective molecule in reducing the melanin levels. The results obtained from the experiments on melanin levels were not in a full correlation with those results obtained from the tyrosinase inhibition tests. This may indicate the probability of the presence of other mechanisms, other than tyrosinase inhibition, involved in the shikimic's whitening efficiency.

We used the fibroblast and melanocytes for the cytotoxicity tests. Results from cytotoxicity effects on fibroblast after 24 hours showed that the cell viability under 200 μM of p-coumaric acid (2), quinic acid (5) and orcinol monohydrate (7), and 500 μM of vanillic acid (3) and shikimic acid (6) were over 80%. The cell viability of above compounds were also over 80% in melanocytes cytotoxicity tests for 48 hours, except quinic acid which had a 76.6 ± 1.3% cell viability. Furthermore, the cell viability under orcinol monohydrate (7) and vanillic acid (3) for fibroblast and melanocytes were higher than the control group after 24 or 48 hours incubation period. Therefore, one can speculate that these two compounds may probably promote cell proliferation.

The whitening efficiency of the compounds was examined by evaluation of tyrosinase inhibitory capacities in the animal model of zebrafish. Anti-oxidant activity was evaluated by DPPH radical scavenging, ABTS+ free radical scavenging.

Experimental results showed that the DPPH free radical scavenging capacity of shikimic acid (6), at 100 μg/ml, was over 95%, and in the ABTS free radical scavenging test, orcinol monohydrate showed a capacity over 95% at the same concentration (data not shown). As for the tyrosinase activity, the inhibition rates of p-coumaric acid (2), orcinol monohydrate (7) and gallic acid were over 50%, and orcinol monohydrate (7) had the best tyrosinase inhibitory activity (93.2±3.4%).

Conclusions

Shikimic acid had an excellent performance as a whitener with strong free radical scavenging ability. Shikimic acid showed low cell toxicity along with good inhibitory effects on

the pigmentation of zebrafish. The whitening effect of shikimic acid is most likely due to its inhibitory potential on tyrosinase activity. Shikimic acid may consider being an excellent candidate to be used as a skin whitening cosmetics additive.

Acknowledgements

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Conflict of Interests

The Authors declare that they have no conflict of interests

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