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The Correlation of *in Vitro* Mushroom Tyrosinase Activity with Cellular Tyrosinase Activity and Melanin Formation in Melanoma Cells A2058

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ABSTRACT

The inhibition of mushroom tyrosinase activity *in vitro* has commonly been employed for evaluation of the whitening effects of various drugs and cosmetic products because tyrosinase is the limiting enzyme in melanin formation in skin. However, questions remain as to whether the *in vitro* inhibition of tyrosinase activity adequately represents the whiting effects since it does not account for cellular uptake of test samples. We therefore evaluated the correlations of the inhibition of cell-free mushroom tyrosinase activity with that of cellular tyrosinase activity and melanin formation in A2058 melanoma cell line using kojic acid, ascorbic acid and water extracts of seven Chinese herbs and seven mixtures of these herbs extracted with water, ethanol or ethyl acetate at different temperatures. No significant correlation was found for the inhibition of: (1) mushroom tyrosinase activity *vs.* cellular tyrosinase activity (r = 0.37, p = 0.25, n = 16) or *vs.* cellular melanin formation (r = 0.32, p = 0.28); and (2) cellular tyrosinase activity vs. cellular melanin formation (r = 0.18, p = 0.43). Ultraviolet A irradiation of melanoma cells A2058 markedly improved the correlation between the inhibition of cellular tyrosinase and of melanin formation of these samples (r = 0.97, p < 0.001). The results suggest that UVA irradiation of melanoma A2058 cells be a useful screening method for evaluating the potential whitening effect of Chinese herbs.

Key words: tyrosinase, melanin, UVA, melanoma cells, whitening effect

INTRODUCTION

Melanin, which plays an important defensive role against the harmful effects of ultraviolet (UV) radiation of sunlight including UVA and UVB, is produced normally by melanocytes and, to a greater extent, by melanoma cells. Overexposure to UV radiation can lead to a pathological increase in melanin production[(1)]. Epidermal hyper-pigmentation results from increased activity of melanogenic enzymes, such as tyrosinase, and results in various dermatological disorders, such as melasma, post-inflammatory melanoderma and solar lentigo[(2)]. Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) is a multifunctional copper-containing enzyme and commonly present in microorganisms, plants and animals. Tyrosinase catalyzes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and of DOPA to DOPA quinone, both reactions being the first two rate-limiting steps in the melanin synthesis pathway[(3)].

Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in preventing pigmentation disorders and other melanin-related health problems in human beings. To treat these problems, depigmenting compounds such as kojic acid, arbutin and others are used[(4,5)]. In traditional Chinese medicine, a whitening effect has been reported for many herbal drugs, which contain effective compounds like oxyresveratrol and gentisic acid, and the depigmentation effects by these compounds may be associated with the inhibition of tyrosinase activity[(6-8)]. Furthermore, tyrosinase inhibitors are important in cosmetic applications for skin whitening effects, because many men and women prefer lighter skin color[(9)]. The whitening effect

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of a compound is commonly measured as the inhibition of mushroom tyrosinase in cell-free systems or the inhibition of cellular tyrosinase activity[10,11]. For example, most melanin synthesis inhibitors, such as kojic acid[4], arbutin[5] and ellagic acid[12] suppress melanogenesis by inhibiting tyrosinase activity. However, little or no evidence exists to demonstrate the correlation of the inhibition of mushroom tyrosinse activity with cellular tyrosinase activity or with the inhibition of cellular melanin formation.

In the present study, we investigated the correlations of three systems, i.e., the *in vitro* inhibition mushroom tyrosinase activity, the inhibition of cellular tyrosinase activity, and the inhibition of cellular melanin formation in A2058 melanoma cell line using the extracts of seven individual Chinese herbs (S1-S7), which have known whitening effects, and seven mixtures of these herbs in fixed proportions (M1-M7), which are known as the "Seven Whitening Pastes" used in ancient Chinese royal families. In addition, kojic acid and ascorbic acid, which are known whitening agents[4,13], were also used for comparison. We also employed UVA irradiation to study the correlations between the inhibition of cellular tyrosinase activity and that of melanin formation in melanoma cells in these samples, as UV-induced melanogenesis is a well known physiological response of human skin exposed to UV radiation.

MATERIALS AND METHODS

I. *Materials*

Human metastatic melanoma cells A2058 (BCRC 60240) were purchased from the Food Industry Research & Development Institute (Hsin Chu, Taiwan). The seven kinds of Chinese herbs, *Atractylodes macrocephala* (bai zhu), *Angelica dahurica var. formosana* (bai zhi), *Bletia formosana* (bai ji), *Radix Ampelopsis* (bai lian), *Asiasari radix* (xi xin; Chinese wild ginger), *Poria cocoa* (bai fu ling) and *Typhonium giganteum Engl.* (bai fu zi), were obtained from Biotechnology Center, KO DA Pharmaceutical Co., LTD. (Taoyuan, Taiwan). Melanin, mushroom tyrosinase, L-tyrosine and L-dopa were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, sodium pyruvate, nonessential amino acids were from Gibco/BRL (Grand Island, NY, USA). Culture dishes and plates were from FALCON (Becton Dickinson, NJ, USA).

II. *Preparation of Water Extract of Chinese Herbs*

Seven kinds of Chinese herbs, as stated in materials were ground under liquid nitrogen and extracted with 10 volumes of water at room temperature for 24 hr, and the extracting procedure was repeated three times. These extracts (designated as S1-S7) were filtered through Whatman No. 2 filter paper, concentrated under reduced pressure. After freeze-drying, extracts S1-S7 were stored at -20°C in sealed containers.

III. *Preparation of Water and Ethanol Extract of "Seven Whitening Paste"*

The seven kinds of Chinese herbs mentioned above were mixed at a weight ratio of 10 : 10 : 10 : 5 : 3 : 3 : 3 (Table 1) to prepare so-called "Seven Whitening Paste" (designated as M1-M7) according to ancient Chinese royal recipes. The mixed formula were extracted with 10 volumes of water at room temperature (M1), 50°C (M2), 75°C (M3) or 100°C (M4) for 24 hr. These crude extracts were then filtered through Whatman No. 2 filter paper and concentrated under reduced pressure. After freeze-drying, the samples (M1-M4) were stored in sealed containers at -20°C. To prepare M5 and M6, the mixed formula was extracted with 10 volumes of 50% and 100% ethanol, respectively, for 24 hr. After filtering through Whatman No. 2 filter paper, the filtrates were concentrated under reduced pressure and freeze-dried. To prepare

Table 1. Preparation methods of individual extracts (S1-S7) and mixed formula (M1-M7) of seven Chinese herbs[a]

Sample	Extraction solvent[b] (10-fold volume)	Extraction temperature
Individual extracts		
S1-S7	water	room temperature
Mixed formula		
M1	water	room temperature
M2	water	50°C
M3	water	75°C
M4	water	100°C
M5	water: ethanol (1:1, v/v)	room temperature
M6	ethanol	room temperature
M7	water, then ethyl acetate (1:1, v/v)	room temperature

[a]The seven Chinese herbs include *Angelica dahurica var. formosana*, *Radix Ampelopsis, Atractylodes macrocephala, Bletia formosana*, *Poria cocoa* (cortex removed), *Typhonium giganteum Engl.* and *Asiasari radix* (leaves removed). The mixed formula was prepared at a weight ratio of 10 : 10 : 10 : 5 : 3 : 3 : 3 of these herbs.

[b]The individual extracts and mixed formula of Chinese herbs were extracted three times with indicated solvents, each for 24 hr. These crude extracts were then filtered through Whatman No. 2 filter paper and concentrated under reduced pressure. After freeze-drying, the samples were stored in closed containers at -20°C.

M7, mixed formula was extracted with 10 volumes of water at room temperature for 24 hr. After filtration, the supernatant was further extracted with ethyl acetate (1:1, v/v), and the ethyl acetate layer was then concentrated to dryness under reduced pressure.

IV. *Assay of Cell-free Mushroom Tyrosinase Activity*

Tyrosinase activity was determined spectrophotometrically, as described elsewhere[14], with minor modifications. In brief, 25 μL of 0.5 mM L-dopa, 25 μL of 10 mM L-tyrosine, 875 μL of 50 mM phosphate buffer (pH 6.5) and 25 μL of the test sample solution were mixed. The amount of dopachrome produced in the reaction mixture was determined against blank (solution without enzyme) at 475 nm in an ELISA (enzyme-linked immunosorbent assay) reader.

The percent inhibition of tyrosinase activity was calculated as follows:

% inhibition = [(A-B)/A × 100], where A = absorbance at 475 nm without test sample, and B = absorbance at 475 nm with test sample.

V. *Cell Culture*

Melanoma cells A2058 were cultured in 10% fetal bovine serum and 90% Dulbecco's modified Eagle's medium with 4 mM L-glutamine containing 1.5 g/L sodium bicarbonate, 100 IU/mL penicillin and 100 μg/mL streptomycin sulfate and 4.5 g/L glucose at 37°C in a humidified atmosphere with 5% CO_2. When cells reached 70-80% confluence, they were treated with 0-500 μg/mL of samples at 37°C for 24 hr. Cells were cultured in 10 cm dishes for melanogenesis experiments, including melanin quantification and tyrosinase activity assay.

VI. *Assay of Cellular Tyrosinase Activity*

Tyrosinase activity was measured as described by Jones *et al.*[15], except that melanoma cells A2058 were cultured in 10 cm dishes. After being treated with an individual sample preparation for 24 hr, cells were washed with potassium phosphate buffered saline (PBS, pH 7.4) and disrupted in PBS containing 1% Triton X-100. Then, cells were lysed by freezing and thawing, and the lysates were centrifuged at 10,000 × g for 10 min. Protein content was determined using Bio-Rad protein assay kit. Each well of a 96-well plate contained approximately 40 μg protein, 2.5 mM L-DOPA, and 0.1 M PBS. After incubation at 37°C for 1 hr, the absorbance (as optical density, OD) was measured at 475 nm in an ELISA reader. Tyrosinase inhibitory activity was calculated using the following formula:

Tyrosinase inhibition (%) = [1 – (OD_{475nm} of sample/$OD_{475\ nm}$ of control)] × 100

VII. *Measurement of Melanin Content in Melanoma Cells*

The melanin content of melanoma cells was determined by the method of Rosenthal *et al.*[16] and Wu *et al.*[10] with minor modification. Melanoma cells A2058 were homogenized in 0.1 M phosphate buffer (pH 7.4) and centrifuged 10,000 g. The resulting pellet was resuspended in 0.01 M sodium carbonate (pH 7.8) and incubated with 0.3% hydrogen peroxide for 30 min at 100°C. The samples were allowed to cool, and the melanin content was measured by the optical absorbance at 450 nm. A melanin standard curve was prepared by dissolving synthetic melanin in 0.01 M sodium carbonate (pH 7.8) and treated as above.

VIII. *UVA-Promoted Melanin Formation*

The method of UVA-promoted melanin formation was employed as described by Shih and Hu[17] with slight modification. Briefly, after incubation with the sample for 24 hr, melanoma cells A2058 were irradiated by UVA at room temperature for 30 min, using a Vilber Lourmat UV lamp (two identical lamps with main output at 365 nm and cut-offs below 315 nm and beyond 400 nm). The surface of the mixture was kept at a distance of 3 cm from the filter surface where the light intensity was 5 mW/cm^2 (with a total energy of 180 kJ/m^2/h or 90 kJ/m^2/30 min), as measured using a Vilber Lourmat radiometer. Cellular melanin contents and tyrosinase activities were measured immediately.

IX. *Statistical Analyses*

Data are expressed as means ± SD and analyzed statistically using one-way ANOVA followed by Duncan's Multiple Range test for comparison of group means. A p value < 0.05 is considered statistically significant.

RESULTS

I. *Effect of Herbal Extracts on Mushroom Tyrosinase Activity, Cellular Tyrosinase Activity and Cellular Melanin Formation*

As shown in Table 2, regardless of the evaluation methods, kojic acid (10 μM) exhibited the best effects, whereas ascorbic acid at the same concentration (10 μM) displayed more potent inhibitory effects on cellular melanin formation (35.9%, $p < 0.05$, as compared to the control) than on mushroom and cellular tyrosinase activities (5.1-14.3%). However, the inhibitory effects of extracts S1-S7 and M1-M7 evaluated at a non-cytotoxic concentration (100 μg/mL) on cell-free mushroom tyrosinase activity and cellular tyrosinase activity as well as cellular melanin formation were quite different. For instance, extracts M1-M4 inhibited cellular tyrosinase activity by 42.9-56.5% but did not significantly affect cellular melanin formation. Furthermore, extracts S7, M3 and M4 had

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Table 2. The inhibitory effects of kojic acid, ascorbic acid, individual extracts (S1-S7) and mixed formula (M1-M7) of seven Chinese herbs on mushroom tyrosinase activity, cellular tyrosinase activity and cellular melanin formation in melanoma cells A2058[a]

Samples	Inhibition (%)		
	Mushroom tyrosinase activity	Cellular tyrosinase activity	Cellular melanin formation
Kojic acid (10 μM)	33.3 ± 4.1[b]	52.7 ± 5.2	52.5 ± 8
Ascorbic acid (10 μM)	5.1 ± 0.2	14.3 ± 7	35.9 ± 3.3
Individual extracts (100 μg/mL)			
S1	6.6 ± 3	-9.6 ± 2	-20.3 ± 10
S2	9.0 ± 3	-21.2 ± 5	-5.8 ± 1
S3	12.2 ± 6	1.9 ± 1	17.4 ± 4
S4	14.0 ± 4	-13.5 ±3	15.9 ± 12
S5	8.1 ± 3	48.1 ± 12	18.8 ± 10
S6	10.1 ± 2	48.1 ±18	17.4 ± 5
S7	11.7 ± 1	36.5 ± 10	-66.7 ±5
Mixed formula (100 μg/mL)			
M1	0.5 ± 3	42.9 ± 9	-6.4 ± 5
M2	-3.0 ± 0	50.0 ± 3	-9.4 ± 8
M3	0.7 ± 1	56.5 ± 6	-31.6 ± 23
M4	1.1 ± 2	53.5 ± 22	-19 ± 6
M5	39.9 ± 6	70.7 ± 5	59.5 ± 23
M6	38.9 ± 1	77.4 ± 5	76.6 ± 11
M7	35.0 ± 11	52.7 ± 24	88.0 ± 4.7

[a]A2058 cells were incubated with an individual extract or a mixed formula at a concentration of 100 μg/mL for 24 h.
[b]Data are presented as means ± SD (n=3).

little or no inhibition on mushroom tyrosinase activity but had significant inhibition (36.5-56.5%) on cellular tyrosinase activity; surprisingly, these samples promoted cellular melanin formation. Most strikingly, extract S7 inhibited mushroom and cellular tyrosinase activity by about 11.7% and 36.5%, respectively, but promoted melanin formation by 66.7% ($p < 0.01$).

As also shown in Table 2, extracts M5 (50% ethanol extract), M6 (100% ethanol extract) and M7 (ethyl acetate extract) exhibited the strongest inhibition on mushroom and cellular tyrosinase activity as well as on cellular melanin formation.

II. *Correlation of Inhibition of Mushroom Tyrosinase Activity vs. Cellular Tyrosinase Activity or vs. Cellular Melanin Formation*

As shown in Figure 1, there was no significant correlation between the inhibition of cell-free mushroom tyrosinase activity and that of cellular tyrosinase activity for the 16 samples, i.e., kojic acid, ascorbic acid, extracts S1-S7 and M1-M7 ($r = 0.37$, $p = 0.25$). In addition, the effects of these samples on the inhibition of mushroom tyrosinase activities also did not significantly correlate to cellular melanin formation ($r = 0.32$, $p = 0.28$). The effects of 16 samples on the inhibition of cellular tyrosinase activity *vs.* cellular melanin formation were significantly correlated ($r = 0.78$, $p < 0.01$) (Figure 2A). However, it was apparent in Figure 2A that the 16 samples were divided into two separate groups: one with 12 samples (as shown in the dashed box) and another with four samples which had the strongest inhibition of cellular tyrosinase activity and melanin formation (i.e., kojic acid, extracts M5, M6 and M7). When kojic acid, extracts M5, M6 and M7 were excluded from Figure 2A, no significant correlation was found ($r = 0.18$, $p = 0.43$) for the remaining 12

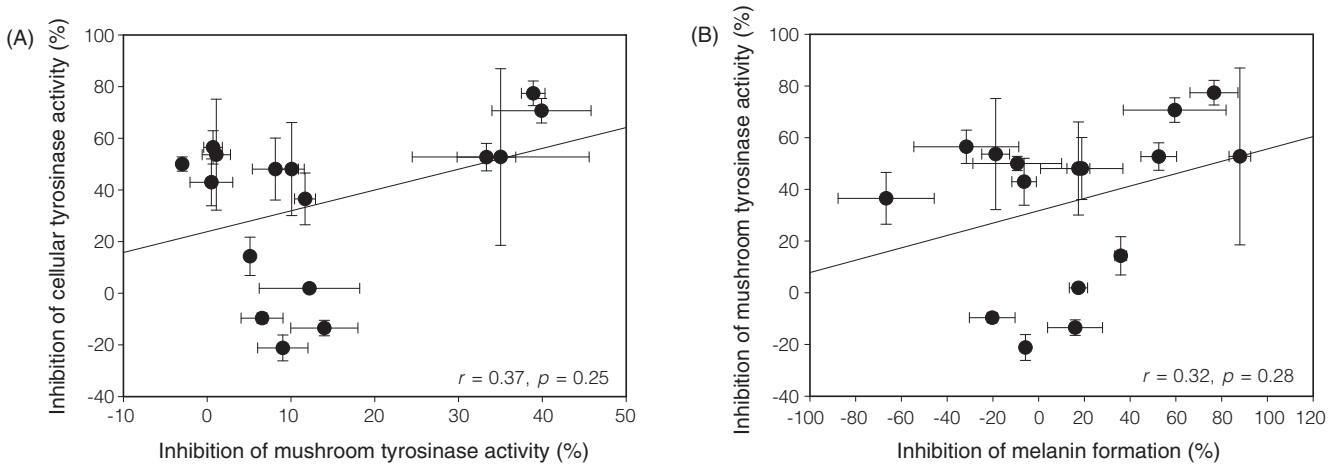

Figure 1. The correlation of the inhibition of mushroom tyrosinase activity *vs.* the inhibition of cellular tyrosinase activity (A) or *vs.* the inhibition of cellular melanin formation (B) in melanoma cells A2058 incubated for 24 h with kojic acid, ascorbic acid, individual extracts (S1-S7) or mixed formula (M1-M7) of seven Chinese herbs. Data are presented as means ± SD (n=3).

samples (Figure 2B). There was also no significant correlation between the inhibition of cellular tyrosinase activity and the inhibition of melanin formation among kojic acid, extracts M5, M6 and M7 (r =0.07, p = 0.93).

III. *Effect of UVA Irradiation on the Correlations of Inhibition of Mushroom Tyrosinase Activity, Cellular Tyrosinase Activity and Melanin Formation in Melanocytes*

We observed that the tyrosinase activity and melanin formation in UVA irradiated melanoma cells A2058 were 830 nmol/mg protein/min and 19.6 μg/10^6 cells, respectively, which were about 4-fold higher than these of untreated cells (187 nmol/mg protein/min and 4.2 μg/10^6 cells, respectively). Table 3 shows the individual effect of the 16 samples on the inhibition of cellular tyrosinase activity and melanin formation in melanoma cells A2058 irradiated with UVA. Overall, the inhibitory effects on cellular tyrosinase activity of the 16 samples were consistent with those of cellular melanin formation, as evident by the highly significant correlation (r = 0.97, p < 0.001) between the inhibition of cellular tyrosinase activity and the inhibition of cellular melanin formation (Figure 3).

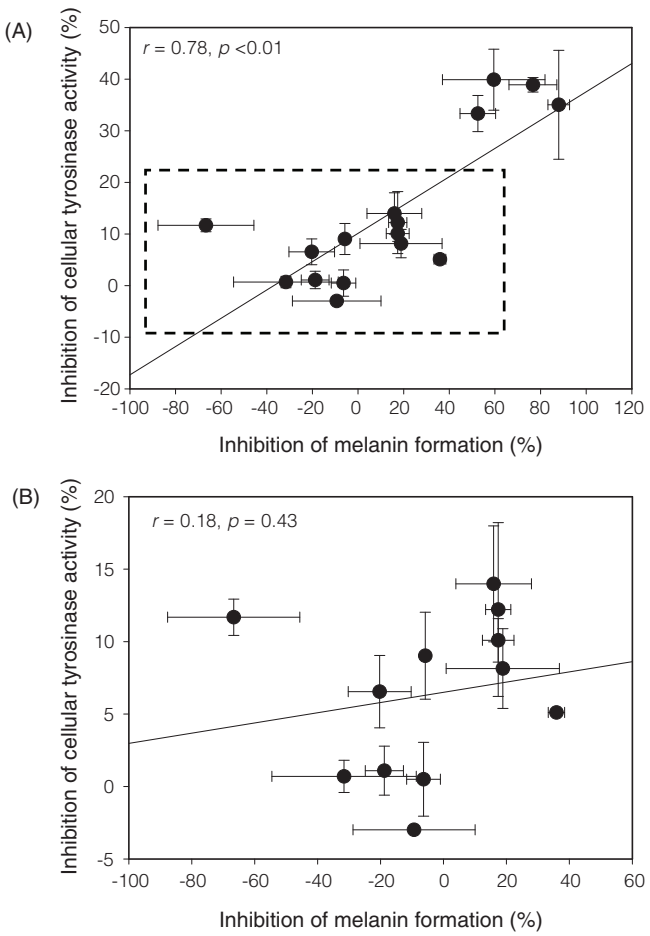

Figure 2. (A) The correlation of inhibition of cellular tyrosinase activity vs. the inhibition of cellular melanin formation in melanoma cells A2058 incubated with kojic acid, ascorbic acid, individual extracts (S1- S7) or mixed formula (M1- M7) of seven Chinese herbs for 24 hr. (B) The correlation of the data points in the dashed box of (A) (12 samples) excluding the four data points (kojic acid, M5, M6 and M7). Data are presented as means ± SD (n=3).

Table 3. The inhibitory effects of kojic acid, ascorbic acid, individual extracts (S1-S7) and mixed formula (M1-M7) of seven Chinese herbs on cellular tyrosinase activity and cellular melanin formation in UVA radiated A2058 cells[a].

Samples	Inhibition (%)	
	Cellular tyrosinase activity	Cellular melanin formation
Kojic acid (10 μM)	82.7 ± 5[b]	62.5 ± 5
Ascorbic acid (10 μM)	34.8 ± 7	21.2 ± 7
Individual extracts (100 μg/mL)		
S1	2.2 ± 2	-3.5 ± 2
S2	3.8 ± 1	-1.9 ± 1
S3	10.9 ± 1	8.6 ± 1
S4	13.5 ± 3	13.5 ± 3
S5	38.1 ± 12	32.5 ± 12
S6	48.1 ± 18	41.1 ± 7
S7	-6.2 ± 10	-2.3 ± 10
Mixed formula (100 μg/mL)		
M1	14.5 ± 9	14.5 ± 9
M2	10.3 ± 3	10.3 ± 3
M3	10.4 ± 6	10.4 ± 6
M4	38 ± 10	20.3 ± 8
M5	79.7 ± 5	69.8 ± 5
M6	60.4 ± 5	64.3 ± 6
M7	70.1 ± 10	71.2 ± 10

[a]A2058 cells pretreated with each test sample for 24 hr were then irradiated with UVA (main output at 365 nm, 180 kJ/m^2) for 30 min.
[b]Data are presented as means ± SD (n=3).

DISCUSSIONS

In this study we investigated the correlations of *in vitro* inhibition of cell-free mushroom tyrosinase activity and the inhibition of cellular tyrosinase activity with the inhibition of cellular melanin formation in melanoma cells A2058 using extracts of seven Chinese herbs (extracts S1-S7), which have known whitening effects,

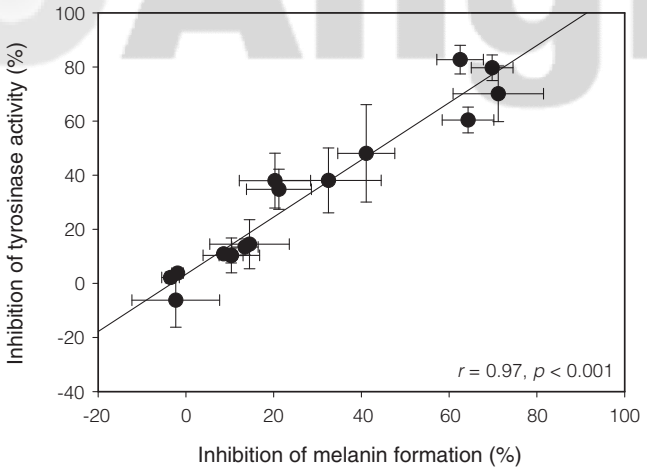

Figure 3. Effect of UVA irradiation (main output at 365 nm, 180 kJ/m^2) on the correlation between inhibition of cellular tyrosinase activity and melanin formation in A2058 cells incubated with kojic acid, ascorbic acid, individual extracts (S1-S7) or mixed formula (M1-M7) of seven Chinese herbs for 24 h. Data are presented as means ± SD (n=3).

and their mixed formula (M1-M7), along with the known whitening agents, kojic acid and ascorbic acid. Except for extracts M6 and M7, which significantly deceased cell viability to 57% and 78%, respectively, at 500 μg/mL, no cytotoxicity was found for the other samples at concentrations up to 500 μg/mL (data not shown).

Although the whitening effect of a compound is commonly measured as the inhibition of mushroom or cellular tyrosinase activity[10,11], little is known about the correlations for the inhibition of mushroom tyrosinse activity with cellular tyrosinase activity or with the inhibition of cellular melanin formation. As revealed from the present study, samples that inhibit mushroom tyrosinase activity in a cell-free system do not necessarily inhibit cellular tyrosinase activity or cellular melanin formation, and vise versa. For example, extracts M1-M4 inhibited cellular tyrosinase activity by 42.9-56.5% but did not significantly affect cellular melanin formation. Furthermore, contradictory results may be obtained from some samples using these evaluation systems; a most striking example was extract S7, which inhibited mushroom and cellular tyrosinase activity by about 11.7% and 36.5%, respectively, but promoted melanin level by 66.7% ($p < 0.01$).

A possible explanation for the poor correlations among these test systems is that the inhibition of melanin formation can involve multidimensional mechanisms, such as direct inactivation of the enzyme tyrosinase by binding with the copper-containing active site of the enzyme, mediation of the switch mechanism from eumelanin to phaeomelanin production, quenching of free radicals and peroxides that contribute to tyrosinase activation and melanin formation, and modulation of depigmenting abilities of melanocytotoxic agents[18]. In addition, Aoki *et al.*[19] have reported that oolong tea extracts inhibit melanogenesis by deceasing intracellular tyrosinase at the mRNA level in B16 mouse melanoma cells, whereas the extracts showed no inhibitory effects on mushroom tyrosinase in a cell-free system. The wide range of anti-melanogenic actions implies that the mere use of inhibition of cell-free mushroom tyrosinase activity for a sample may be insufficient for the evaluation of its whitening effect.

Another factor that may affect the inhibitory effects of the test samples is the nature of the extracts, as we found that the mixed formula extracted by organic solvents (i.e., extracts M5-M7) exerted stronger inhibitory effects than their water-extract counterparts (i.e., extracts M1-M4). It is conceivable that the organic-solvent extracts of these samples are probably able to enter the cells more readily to achieve the inhibition of cellular tyrosinase activity and melanin formation. Indeed, it has been demonstrated that the biological activities (e.g., antioxidant activity and anti-tyrosinase activity) of extracts from plants depend on the extraction solvents and extraction techniques used[11, 20].

In order to improve the evaluation methods for the potential whitening effects of natural products, we applied UVA irradiation on melanoma cells A2058, as several reports have demonstrated the inhibitory effects of various samples on tyrosinase activity and melanin formation in UV-irradiated cells[21-23]. Indeed, we found that UVA irradiation strongly promoted the correlation ($r = 0.97$, $p < 0.001$, n = 16) of inhibition of cellular tyrosinase activity with the inhibition of cellular melanin formation. The effect of UVA irradiation may be related to its activation of cellular tyrosinase. In this regard, Yanase *et al.*[24] have shown increased tyrosinase mRNA expression followed by increased amount of tyrosinase protein in UVA induced melanogenesis in cultured normal human epidermal melanocytes. Ramirez-Bosca *et al.*[25] also have found a marked increase in tyrosinase activity and melanin content after irradiation with UVA/B at 200 mJ/cm^2 in melanocytes.

CONCLUSIONS

The present study demonstrates that the inhibition of mushroom tyrosinase activity or that of cellular tyrosinase activity does not represent a useful marker for cellular melanin formation. The results further suggest that the use of UVA irradiation to enhance tyrosinase activity and melanin content in melanoma cells is more appropriate for the evaluation of potential whitening effects for natural and herbal products. However, the final proof for whitening effects of any product should await the data from animal or human studies.

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