A novel bioactive chalcone of *Morus australis* inhibits tyrosinase activity and melanin biosynthesis in B16 melanoma cells

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Abstract: The methanol extract of *Morus australis* (shimaguwa) acts as a whitening agent due to the inhibition of tyrosinase activity. In order to explore the mechanism(s) of the whitening action, constituents of the 95% methanol extract from the dried stems of shimaguwa were isolated and their skin-whitening capacity was examined. Bioassay-guided fractionation of the methanol soluble extract of shimaguwa led to the isolation of 2, 4, 2', 4'-hydroxycalcone (chalcone 1) and three analogues of chalcone 1 with 3'-substituted resorcinol moieties (chalcones 2-4). Chalcone derivative 4 proved to be a novel compound and was fully characterized. Chalcones 1-4 were evaluated for inhibition activity on mushroom tyrosinase using L-tyrosine as the substrate. The parent chalcone 1 was a highly effective inhibitor of tyrosinase activity ($IC_{50} = 0.21 \mu M$) compared to arbutin ($IC_{50} = 164 \mu M$). Compared to chalcone 1, chalcones 2 and 3, which possess 3'-substituted isoprenyl or bulky 2-benzoylbiphenyl, showed significantly decreased tyrosinase activity, while chalcone 4, possessing 3'-substituted 2-hydroxy-1-pentene group, showed slightly increased activity. The effects of chalcones 1-4 on melanin synthesis, without affecting cell growth, were assayed in melanin-producing B16 murine melanoma cells. Chalcone 3 significantly reduced cell viability before reaching the IC_{50} value for melanin synthesis. In contrast, the inhibitory effects of chalcones 1, 2 and 4 were more than 100-fold greater than that of arbutin, with little or no cytotoxicity. More significantly, chalcone 2, which exhibited less tyrosinase inhibitory activity compared to the parent chalcone 1, showed the highest inhibition of melanin synthesis in B16 cells among the chalcones tested. Accordingly, chalcones 1 and 2, and the novel chalcone 4 might be the active components responsible for the whitening ability of shimaguwa. Moreover, whitening ability was not exclusively due to tyrosinase inhibition.

Key words: *Morus australis*, Shimaguwa, Chalcone, Tyrosinase, Melanin synthesis

1 INTRODUCTION

Melanin is related to the colour of the skin and hair, and its major role is protection against UV light^{1,2)}. However, an excessive accumulation of melanin causes hyperpigmentation such as melasma, post-inflammatory melanoderma and solar lentigo^{3,4)}. Melanin is synthesized in mammals in the melanosomes of melanocytes. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase related protein 2 (TRP-2)⁵⁾. Tyrosinase is the rate-limiting enzyme for melanogenesis and catalyzes the hydroxylation of L-tyrosine to 3, 4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone, these being the first stages of

melanin synthesis⁶⁾. Accordingly, inhibition of tyrosinase activity prevents hyperpigmentation and leads to skin whitening.

Despite the large number of tyrosinase inhibitors reported, their identification and isolation from natural sources is currently one of the most important undertakings⁷⁾. Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at a reasonably low cost, especially when rich sources are identified. In addition, tyrosinase inhibitors are becoming increasingly important in the cosmetic industry due to their skin-whitening effects⁸⁾. These facts led us to focus our research on the exploration of natural tyrosinase inhibitors.

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Morus australis, known as shimaguwa in Japan, is a deciduous tree or shrub that grows naturally in Asian countries⁹⁾. The root, bark, and stem have several medicinal usages in Japanese traditional medicine, such as in the treatment of diabetes, arthritis and rheumatism¹⁰⁾. In addition, as part of our continuing search for tyrosinase inhibitory agents of natural origin, the crude methanol extract from the dried stems of shimaguwa was found to have potent inhibitory activity against mushroom tyrosinase. Therefore, shimaguwa has great potential as a highly effective skin-whitening agent, and various products containing shimaguwa extract have been manufactured and marketed. However, details of the shimaguwa constituents involved in the inhibitory activity have not yet been investigated, and available information on the skin-whitening properties of the shimaguwa extracts remains limited. We thus attempted to isolate the constituents of shimaguwa and to characterize its skin-whitening properties.

In this study, bioassay-guided fractionation of the methanol soluble extract of shimaguwa led to the isolation of three known, and one novel, compounds with strong tyrosinase inhibitory activity. We also evaluated the inhibitory effects of these active components on melanin synthesis in B16 murine melanoma cells. This is the first report characterizing the skin-whitening properties of compounds extracted from *Morus australis*.

2 EXPERIMENTAL

2.1 Chemicals and materials

Tyrosinase (EC 1.14.18.1, 50000 units) from mushroom and arbutin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). L-Tyrosine was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan).

Eagle's minimal essential medium (EMEM) and fetal bovine serum (FBS) for melanocyte cultivation were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Invitrogen Co. (Tokyo, Japan), respectively.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was purchased from Promega Co. (Tokyo, Japan) and used for cell proliferation assay. All other chemicals and reagents were purchased from Wako Pure Chemical Industries Ltd.

2.2 Fractionation and isolation of inhibitory compounds from shimaguwa

The stems of *M. australis* (shimaguwa) were collected from the University of the Ryukyus campus (Okinawa, Japan). To prepare the inhibitory compounds, the fractionation from stems of shimaguwa was carried out as described in Fig. 1.

Dried shimaguwa stem (400 g) was ground using a mini-grinder (Model: DF-15, Shenzhen Laitong Company, Shenzhen, PR China) and packed into a paper extraction bag.

Fig. 1 Isolation scheme for chalcone 1-4 from *Morus australis* (shimaguwa).

Extraction was performed with 95% methanol using a Soxhlet apparatus for 3 h. The extract was concentrated and suspended in water, and then further extracted with EtOAc and n-BuOH continuously. Those two fractions and the remaining water fraction were concentrated under reduced pressure. Since the EtOAc fraction exhibited the highest tyrosinase inhibitory activity, this fraction was subjected to column chromatography (CC; 30 mm i.d. $\times$ 450 mm) over silica gel (Wakogel C-100, Wako Pure Chemical Industries Ltd.) using hexane/EtOAc [10:0, 8:2, 6:4, 4:6, 2:8 and 0:10] mixtures to give six fractions. Since three obtained fractions [Hexane-EtOAc (4:6, 2:8 and 0:10)] retained inhibitory activity (data not shown), the fractions were then combined and were divided into fraction 1-8 in a Toyopearl HW-40C column (25 $\times$ 650 mm, Tosoh Corporation, Tokyo, Japan) [MeOH-H₂O (80:20 $\rightarrow$ 100:0)]. Fraction 4 was subjected to LiChroprep RP-8 column chromatography (Merck Chemicals, Germany) [MeOH-H₂O (40:60 $\rightarrow$ 100:0)] to give three fractions (fr. 4.1-4.3); fr. 4.2 was re-subjected to a combination of Toyopearl HW-40C column with 70% MeOH and LiChroprep RP-18 column chromatography (Merck Chemicals) with 50% MeOH including 0.1% formic acid to give chalcone **1** (5.3 mg). Fraction 4.3 was subjected to preparative HPLC [ODS, MeOH-H₂O-HCOOH (70:30:0.1)] to give chalcone **2** (8.0 mg). Fraction 5 was subjected to a combination of LiChroprep RP-8 column chromatography [MeOH-H₂O-HCOOH (50:50:0.1 $\rightarrow$ 100:0:0.1)] and LiChroprep RP-18 column chromatography [MeOH-H₂O-HCOOH (40:60:0.1 $\rightarrow$ 100:0:0.1)] to give chalcone **3** (3.4 mg). Fraction 7 was subjected to a combination of LiChroprep RP-8 column chromatography [MeOH-H₂O-HCOOH (80:20:0.1 $\rightarrow$ 100:0:0.1)] and LiChroprep RP-18 column chromatography [MeOH-H₂O-HCOOH (75:25:0.1 $\rightarrow$ 80:20:0.1)] to give chalcone **4** (6.8 mg). All of the isolated chalcones were identified on the basis of the following spectroscopic data.

2, 4, 2', 4',-Tetrahydroxychalcone (chalcone 1): UV $\lambda_{\max}$ nm: 257 (sh), 315 (sh), 388. ¹H-NMR (500 MHz, acetone-*d*₆) δ 8.82 (1H, d, *J* = 15.4 Hz), 8.01 (1H, d, *J* = 9.0 Hz), 7.80 (1H, d, *J* = 15.4 Hz), 7.73 (1H, d, *J* = 8.7 Hz), 6.51 (1H, d, *J* = 2.3 Hz), 6.45 (1H, d, *J* = 2.3 Hz), 6.43 (1H, d, *J* = 2.3 Hz), 6.34 (1H, d, *J* = 2.3 Hz). ¹³C-NMR (125 MHz, acetone-*d*₆) δ 193.2, 167.5, 165.5, 162.4, 160.0, 141.0, 132.9, 131.7, 117.3, 115.2, 109.1, 108.6, 103.8. Negative ESI-MS *m/z* 271.4 [M - H]⁻.

2, 4, 2', 4',-Tetrahydroxy-3'-(3"-methyl-2"-butenyl)-chalcone (chalcone 2): UV $\lambda_{\max}$ nm: 388. ¹H-NMR (500 MHz, acetone-*d*₆) δ 8.17 (1H, d, *J* = 15.5 Hz), 7.84 (1H, d, *J* = 8.9 Hz), 7.75 (1H, d, *J* = 15.6 Hz), 7.64 (1H, d, *J* = 8.6 Hz), 6.47 (1H, d, *J* = 7.8 Hz), 6.46 (1H, d, *J* = 2.5 Hz), 6.41 (1H, dd, *J* = 8.4, 2.1 Hz), 5.23 (1H, dt, *J* = 7.2, 1.3 Hz), 3.32 (1H, d, *J* = 7.2 Hz), 1.73 (3H, s), 1.59 (3H, s). ¹³C-NMR (125 MHz, acetone-*d*₆) δ 193.4, 165.1, 162.4, 162.2, 159.9, 140.7, 131.7, 131.3, 129.9, 123.3, 117.5, 116.1, 115.2, 114.5, 109.1,

107.8, 103.6, 25.8, 22.2, 17.8. Negative ESI-MS *m/z* 338.9 [M - H]⁻.

1-[3'-(4"-[10", 12"-Dihydroxy-11"-(3'''-methyl-2'''-butenyl)benzoyl]-5"-(16", 18"-dihydroxyphenyl)-1"-methyl-2"-cyclohexene-3"-yl]-2', 4'-dihydroxyphenyl]-3-(5,7-dihydroxyphenyl)-[3"R-[3" α (*E*), 4" α , 5" β]]-2-propene-1-one (chalcone 3): UV $\lambda_{\max}$ nm: 318. ¹H-NMR (500 MHz, methanol-*d*₄) δ 8.38 (1H, d, *J* = 9.1 Hz), 8.14 (1H, d, *J* = 15.4 Hz), 7.85 (1H, d, *J* = 9.1 Hz), 7.71 (1H, d, *J* = 15.5 Hz), 7.65 (1H, d, *J* = 8.6 Hz), 6.97 (1H, d, *J* = 8.4 Hz), 6.54 (1H, d, *J* = 2.4 Hz), 6.49 (1H, d, *J* = 2.2 Hz), 6.43 (1H, d, *J* = 9.0 Hz), 6.42 (1H, d, *J* = 8.4 Hz), 6.35 (1H, d, *J* = 9.0 Hz), 6.31 (1H, d, *J* = 8.4 Hz), 5.69 (1H, s), 5.17 (1H, m), 4.67 (1H, t, *J* = 4.6 Hz), 4.14 (1H, s), 3.78 (1H, m), 3.25 (2H, d, *J* = 7.2 Hz), 2.50 (1H, s), 2.43 (1H, s), 1.94 (3H, s), 1.72 (3H, s), 1.59 (3H, s). ¹³C-NMR (125 MHz, methanol-*d*₄) δ 209.5, 193.3, 165.7, 164.6, 163.5, 163.4, 162.3, 159.9, 157.9, 156.4, 140.1, 134.7, 132.1, 131.7, 131.5, 130.7, 128.7, 123.5, 121.7, 117.4, 117.3, 116.2, 115.9, 115.2, 114.0, 113.3, 110.1, 109.1, 108.2, 107.5, 103.6, 103.6, 47.4, 36.4, 32.6, 32.3, 25.8, 23.8, 22.2, 17.8. Positive ESI-MS *m/z* 701.3 [M + Na]⁺. Negative ESI-MS *m/z* 677.3 [M - H]⁻.

3'-(*E*)-4"-hydroxymethyl-2"-butenyl]-2, 4, 2', 4'-tetrahydroxychalcone (chalcone 4): UV $\lambda_{\max}$ nm: 388. ¹H-NMR (500 MHz, acetone-*d*₆) δ 8.20 (1H, d, *J* = 15.5 Hz), 7.89 (1H, d, *J* = 8.9 Hz), 7.78 (1H, d, *J* = 15.4 Hz), 7.68 (1H, d, *J* = 8.6 Hz), 6.54 (1H, d, *J* = 9.0 Hz), 6.52 (1H, brs), 6.45 (1H, dd, *J* = 8.4, 2.2 Hz), 5.52 (1H, dt, *J* = 7.2, 1.3 Hz), 3.89 (2H, s), 3.41 (2H, d, *J* = 7.2 Hz), 1.80 (3H, s). ¹³C-NMR (125 MHz, acetone-*d*₆) δ 193.5, 165.2, 162.6, 162.3, 160.0, 140.9, 136.1, 131.8, 130.0, 123.0, 117.5, 115.8, 115.3, 114.5, 109.2, 107.9, 103.7, 68.5, 21.8, 13.8. Positive ESI-MS *m/z* 379.1 [M + Na]⁺. Negative ESI-MS *m/z* 355.0 [M - H]⁻.

2.3 General procedure

UV spectroscopic data was obtained by high-performance liquid chromatography (HPLC) with a photo-diode array detector (Shimadzu Co., Kyoto, Japan) to monitor all wavelengths from 190 to 370 nm. ¹H-NMR and ¹³C-NMR, including HMQC and HMBC experiments, were recorded by a Jeol A-500 NMR spectrometer (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan) at 500 MHz for ¹H and 125 MHz for ¹³C. Chemical shifts are noted on a δ (ppm) scale with the solvent peaks of δ 2.04 for ¹H and δ 29.3 and 206.3 for ¹³C in acetone-*d*₆. Electro-spray ionization mass spectra (ESI-MS) were recorded by an Esquire 3000 plus (Agilent Technologies, Inc., USA). The ESI-MS conditions were as follow: positive or negative ion mode; injection flow rate, 2 μ L/min; drying gas (nitrogen) flow rate, 4 L/min; nebulizer pressure, 10 psi (= 29.3 kgf/cm²); drying gas temperature, 300°C; mode scan, 50-1000 *m/z*.

2.4 Assay of tyrosinase inhibitory activity

The assay was performed according to the procedure of

Mason *et al.* with slight modification¹¹⁾. Briefly, the test compound solution (10 μ L), dissolved in methanol to generate the final concentrations 0.01–100 μ M, was mixed with 180 μ L of 0.05 M phosphate buffer (pH 6.8) containing 0.5 mM of L-tyrosine in 96-well microplates. After the mixture was pre-incubated at 30°C for 10 min, 10 μ L of tyrosinase solution (9.05 units/mL) was added to the phosphate buffer, mixed, and further incubated at 30°C for 15 min. Methanol without test compounds was used as the control, and arbutin was used as the positive control (1–100 μ M as final concentrations). The absorbance at 470 nm of the mixture was measured by a microplate reader (Benchmark Plus, Bio-Rad, USA).

Data were expressed in terms of tyrosinase inhibitory activity (%), which was calculated according to equation 1:

$$\text{Inhibitory activity (\%)} = [1 - (S - B)/C] \times 100, \quad (1)$$

where *S* is the absorbance of the test compound, *B* is the absorbance of the blank, and *C* is the absorbance of the control.

2.5 Cell culture

Murine B16 melanoma cells (B16 cells) were purchased from Japan Health Science Foundation (Tokyo, Japan). B16 cells were cultivated in EMEM supplemented with 10% FBS under a humidified atmosphere at 37°C and 5% CO₂. Then, the cells were assayed for cell viability and melanin synthesis as described below.

2.6 Assay of cell viability

B16 cells were seeded on 96-well microplates (5 × 10⁴ cells/well) and cultivated as above. After 24 h, the medium (200 μ L) was changed to EMEM containing 10% FBS supplemented with the test compounds or arbutin solution dissolved in methanol (within 0.5% (v/v) methanol as the nontoxic condition), and the cells were cultivated for a further 72 h. In this assay, the final concentration of each supplement was fixed at 1, 5, 10, 50, 100 and 500 μ M. The control cells were cultivated in EMEM (200 μ L) containing 10% FBS without the above supplements.

After cultivation, cell viability was determined by MTS assay. Twenty microlitres of MTS solution (1.9 mg/mL) was added to each well, and the cells were incubated at 37°C and 5% CO₂ for 60 min. After incubation, the absorbance at 490 nm of the cells was determined by a microplate reader (Benchmark Plus).

Data were expressed in terms of cell viability (%), which was calculated according to equation 2:

$$\text{Cell Viability (\%)} = (S - B_s)/(C - B_c) \times 100 \quad (2)$$

where *S* is the absorbance with the supplemented medium, *B_s* is the absorbance with the supplemented medium without cells, *C* is the absorbance with the control medium, and *B_c* is the absorbance of the medium itself (without

cells).

2.7 Assay of melanin synthesis

B16 cells were seeded at a concentration of 5 × 10⁵ cells/dish (60 mm ϕ × 15 mm high) and cultivated as above. After 24 h, the medium was changed to EMEM containing 10% FBS supplemented with the test compounds or arbutin, and the cells were cultivated for a further 72 h. In this assay, methanol without test compounds was used as the control, and arbutin was used as the positive control. A final concentration of test compounds and arbutin reflecting cell viability of greater than 80% was used as the non-toxic condition with respect to melanin synthesis in B16 cells. After cultivation, the colour of the cell pellets was evaluated visually, and the pellets containing a known number of cells were dissolved in 1 N NaOH solution and then sonicated for 1 h. After sonication, the amount of melanin content was then monitored by a microplate reader (Benchmark Plus). Data were expressed in terms of melanin synthesis inhibitory activity (%) compared to the control. The inhibitory activity (%) was calculated according to equation 3:

$$\text{Inhibitory activity (\%)} = [1 - (S - B)/C] \times 100, \quad (3)$$

where *S* is the absorbance with the supplemented medium, *B* is the absorbance with the blank medium, *C* is the absorbance with the control medium.

2.8 Statistical analysis

The results are expressed as mean $\pm$ standard error (SD). The 50% inhibitory concentration (IC₅₀) was determined using a semi-log plot of percentage of the activity versus each test compound concentration.

3 RESULTS AND DISCUSSION

3.1 Structures of compounds

As part of our continuing search for tyrosinase inhibitory agents of natural origin, the crude methanol extract from the dried stems of shimaguwa was found to have potent inhibitory activity against mushroom tyrosinase (data not shown). To further assess compounds possessing potential tyrosinase inhibitory activity from the methanol-soluble extract of dried shimaguwa stem, bioassay-guided fractionation of the extract was carried out. The EtOAc-soluble fraction of the methanol extract, after separation by chromatography, yielded four chalcones (1–4). Chalcones 1–3 were known compounds and identified by their spectroscopic data as 2, 4, 2', 4'-tetrahydroxychalcone (chalcone 1); 2, 4, 2', 4'-tetrahydroxy-3'-[(3"-methyl-2"-butenyl)-chalcone (chalcone 2) and 1-[3'-[4"-[10", 12"-dihydroxy-11"-[(3'''-methyl-2'''-butenyl) benzoyl]-5"-[(16", 18"-dihydroxyphenyl)-1"-methyl-2"-cyclohexene-3"-yl]-2', 4'

Fig. 2 The structures of isolated chalcones from *Morus australis* (shimaguwa). Values are expressed as mean $\pm$ S.D. ($n = 3$).

-dihydroxyphenyl]-3-(5,7-dihydroxyphenyl)-[3'' R-[3'' α (*E*), 4'' α , 5'' β]-2-propene-1-one (chalcone **3**) (Fig. 2). On the other hand, the configuration of chalcone **4** was determined for the first time in this study, and the structural elucidation of the new compound is detailed below.

Chalcone **4** was obtained as an amorphous yellow powder and its UV spectrum was similar to the spectrum of chalcone **2**. In addition, the ^1H - and ^{13}C -NMR spectral data in acetone- d_6 for chalcone **4** was very similar to those for chalcone **2**, except for the chemical shifts at H-4'', H-5'' and C-4'', C-5'' (Table 1). These results suggested that chalcone **4** is based on the chalcone **2** skeleton with one hydroxyl group at C-4'' or C-5''. The location of the group was also confirmed using the HMBC technique (Fig. 3). The H-2'' exhibited correlation with not C-5'' but C-4'', which allowed assignment of the C-4'' position of the group. Moreover, chalcone **4** gave a molecular ion peak $[\text{M} + \text{Na}]^+$ at m/z 379.1 in positive ESI-MS and $[\text{M} - \text{H}]^-$ at m/z 355.0 in negative ESI-MS, together with 20 carbons in the ^{13}C -NMR spectrum, suggesting its molecular formula as $\text{C}_{20}\text{H}_{20}\text{O}_6$. Therefore, this formula showed the group at C-4'' in chalcone **4** was a hydroxyl group. Thus, the structure of the new chalcone **4** was assigned as 3'-[(*E*)-4''-hydroxymethyl-2''-butenyl]-2, 4, 2', 4'-tetrahydroxychalcone.

3.2 Tyrosinase inhibitory activity

Tyrosinase is a rate-limiting enzyme involved in melanin synthesis. Many melanin synthesis inhibitors reduce melanogenesis by directly inhibiting tyrosinase activity⁵. The effect of the obtained four chalcones on tyrosinase activity

Table 1. ^1H - and ^{13}C -NMR data for chalcones **2** and **4**.

Position	2		4	
	^1H δ (multiplicity, J (Hz))	^{13}C δ	^1H δ (multiplicity, J (Hz))	^{13}C δ
1	—	115.2	—	115.3
2	—	159.9	—	160.0
3	6.46 (1H, d, $J = 2.5$)	103.6	6.52 (1H, brs)	103.7
4	—	162.2	—	162.6
5	6.41 (1H, dd, $J = 8.4, 2.1$)	109.1	6.45 (1H, dd, $J = 8.4, 2.2$)	109.2
6	7.64 (1H, d, $J = 8.6$)	131.7	7.68 (1H, d, $J = 8.6$)	131.8
α	7.75 (1H, d, $J = 15.6$)	140.7	7.78 (1H, d, $J = 15.4$)	140.9
β	8.17 (1H, d, $J = 15.5$)	117.5	8.20 (1H, d, $J = 15.5$)	117.5
C=O	—	193.4	—	193.5
1'	—	116.1	—	115.8
2'	—	162.4	—	162.3
3'	—	114.5	—	114.5
4'	—	165.5	—	165.2
5'	6.47 (1H, d, $J = 7.8$)	107.8	6.54 (1H, d, $J = 9.0$)	107.9
6'	7.84 (1H, d, $J = 8.9$)	129.9	7.89 (1H, d, $J = 8.9$)	130.0
1''	3.32 (2H, d, $J = 7.2$)	22.2	3.41 (2H, d, $J = 7.2$)	21.8
2''	5.23 (1H, dt, $J = 7.2$ and 1.3)	123.3	5.52 (1H, dt, $J = 7.2, 1.3$)	123.0
3''	—	131.3	—	136.1
4''	1.73 (3H, s)	25.8	3.89 (2H, s)	68.5
5''	1.59 (3H, s)	17.8	1.80 (3H, s)	13.8

Fig. 3 HMBC correlations observed for chalcone 4.

was examined, with an aim to tentatively evaluate their anti-melanogenesis properties. The relative activity of each chalcone was assayed at different concentrations and expressed as IC_{50} values. Arbutin is a well-known tyrosinase inhibitor¹²⁾, and was thus used as the positive control. As shown in Table 2, the IC_{50} values of chalcones 1-4 were 0.21, 0.82, 4.62 and 0.17 μ M, respectively. The activity of each chalcone was stronger than that of arbutin (IC_{50} = 164 μ M). It is known that the presence of resorcinol in several flavonoids plays a dominant role in tyrosinase inhibition^{13,14)}. Specifically, several chalcones constructed with a resorcinol moiety on aromatic rings, at hydroxyl positions 2, 4 and/or 2', 4', enhance the inhibition compared to chalcone (no hydroxyl group), because the 2, 4-hydroxyl resorcinol subunit strongly chelates the copper ions that act as the active site of tyrosinase¹⁵⁾. Structural analysis of chalcones 1-4 indicated that each chalcone molecule was constructed with resorcinol moieties on both rings A and B, at the hydroxyl group positions 2, 4 and 2', 4' (Fig. 2). This result might indicate that chalcones 1-4 showed strong tyrosinase inhibitory activity as a result of the strong chelation of both 2, 4- and 2', 4'-hydroxyl resorcinol moieties toward tyrosinase copper ions. In fact, chalcone 1 is known to be a safe

and effective tyrosinase inhibitor¹⁵⁾.

Chalcones 2, 3 and 4 are analogues of chalcone 1 with 3'-substituted resorcinol moieties (Fig. 2). Chalcone 3 showed 22-fold lower activity than chalcone 1, in view of the IC_{50} value. This was probably due to the decreased chelating affinity caused by the bulkiness of the 2-benzoylbi-phenyl group at C-3'. Interestingly, chalcone 4 exhibited stronger activity than chalcone 1, whereas chalcone 2 was also less active. It appeared that the 4-hydroxy-1-pentene group at C-3' in chalcone 4 enhanced the activity. In spite of the differences in activity among the four types of chalcones, the activity of each chalcone was much higher than that of the known potent tyrosinase inhibitor, arbutin. Therefore, chalcones 1-4 were further evaluated.

3.3 Melanin synthesis inhibitory activity

A recent study demonstrated that the inhibitory activity of compounds towards mushroom tyrosinase is correlated with their inhibition of melanin synthesis in melanocytes *in vitro*¹⁶⁾. In this study, B16 cells were used because they produce melanin, contain melanogenesis-associated tyrosinase, and are easy to culture *in vitro*¹⁷⁾. We then evaluated the effects of chalcones 1-4 on cell viability and melanin synthesis in B16 cells, with an aim to tentatively evaluate the skin-whitening properties. Here, the relative activity of each chalcone was assayed at different concentrations and expressed as IC_{50} values. Arbutin was used as a positive control in this study because it is a well-known inhibitor of melanin synthesis in B16 cells¹⁸⁾.

The effects of chalcones 1-4 on melanin synthesis and cell viability in B16 cells are shown in Table 2. Chalcone 3 significantly reduced cell viability before reaching the IC_{50} value for melanin synthesis. Hence, chalcone 3 was not included in further melanin synthesis evaluation due to its great cytotoxicity in B16 cells. In contrast, the inhibitory effects of chalcones 1, 2 and 4 were more than 100-fold greater than that of arbutin, with little or no cytotoxicity. In fact, chalcone 1, 2, and 3 were found to visibly suppress

Table 2. Effects of chalcones from *Morus australis* on mushroom tyrosinase activity and melanin synthesis in B16 murine melanoma cells.

Compound	Tyrosinase ^a IC_{50} (μ M)	Melanin synthesis IC_{50} (μ M)	Cell viability (% vs. control) ^b
1	0.21	5.0	103.8
2	0.82	3.8	83.4
3	4.62	N.D. ^c	N.D. ^c
4	0.17	4.0	84.9
Arbutin	164	500	86.9

^a Substrate: L-tyrosine.

^b Cell viability (%) at the IC_{50} for melanin production in B16 cells.

^c Not detected

the formation of B16 cell coloration compared to that of arbutin (data not shown).

Previously, tyrosinase inhibitory activity evaluated *in vitro* has been reported to be important for the investigation of melanin synthesis inhibition in melanocytes¹⁶⁾. An increase in tyrosinase inhibitory activity was associated with increased inhibition of melanin synthesis in B16 cells. Indeed, chalcone **4** showed higher melanin synthesis inhibitory activity in B16 cells than chalcone **1** (IC_{50} = 4.0 and 5.0 μ M, respectively). However, chalcone **2** showed higher melanin synthesis inhibitory activity (IC_{50} = 3.8) than both chalcones **1** and **4**, whereas its tyrosinase inhibitory activity was more than 4-fold lower than that of chalcones **1** and **4**. These results suggested that chalcone **2**, with isoprenyl-substituted moieties, inhibit melanin synthesis in B16 cells not only by inhibition of tyrosinase activity but also by other mechanisms involved in melanogenesis. Arung *et al.* reported that the depigmenting mechanism of flavonoids with isoprenyl-substituted moieties is responsible for the suppression of some pigmenting signals, such as TRP-1 and TRP-2, that function to stimulate melanogenesis, rather than the inhibition of tyrosinase activity¹⁹⁾. This implies that the administration of chalcones **1** and **2**, and the novel chalcone **4** might accelerate the inhibition of melanin synthesis using different anti-melanogenesis approaches, without cytotoxic effects, and may have great potential as a skin-whitening ingredient compared to commercial products such as arbutin.

4 CONCLUSION

In the present work, we attempted to determine the mechanism(s) of the whitening action of *M. australis* (shimaguwa) for application to the development of practical depigmentation drugs and cosmetics. In this study, three known chalcones: 2, 4, 2', 4',-tetrahydroxychalcone (chalcone **1**), 2, 4, 2', 4',-tetrahydroxy-3'-(3"-methyl-2"-butenyl)-chalcone (chalcone **2**) and 1-[3'-[4"-[10", 12"-dihydroxy-11"- (3'''-methyl-2'''-butenyl) benzoyl]-5"- (16", 18"-dihydroxyphenyl)-1"-methyl-2"-cyclohexene-3"-yl]-2', 4'-dihydroxyphenyl]-3-(5,7-dihydroxyphenyl)-[3" R-[3" α (E), 4" α , 5" β]]-2-propene-1-one (chalcone **3**), and one novel chalcone: 3'-[(E)-4"-hydroxymethyl-2"-butenyl]-2, 4, 2', 4'-tetrahydroxychalcone (chalcone **4**) were isolated from bioassay-guided fractionation of the 95% methanol soluble extract of shimaguwa. The structures of these chalcones were determined on the basis of spectroscopic evidence. We also investigated the effect of chalcones **1-4** on tyrosinase inhibition, as well as on melanin synthesis inhibition in B16 cells, from the viewpoint of skin-whitening properties. Interestingly, all chalcones tested significantly inhibited tyrosinase activity compared to arbutin. Moreover, chalcones **1**, **2** and **4** showed

stronger melanin synthesis inhibitory activity than arbutin, with little or no cytotoxicity, via not only inhibition of tyrosinase activity but also other mechanisms involved in melanogenesis. Therefore, further biochemical studies of melanogenic protein expression in B16 cells on chalcones **1**, **2** and **4** should be undertaken to shed light on other properties involved in melanogenesis inactivation. Chalcones **1** and **2**, and the novel chalcone **4**, isolated from shimaguwa, may have great potential as an effective skin care formulation, especially for skin whitening.

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