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Melanin Synthesis Inhibitors from *Balanophora fungosa*

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ABSTRACT: Tyrosinase, trypsin, and tryptase are known to play important roles in melanin production of human skin. This paper describes the study of the inhibitory effect of *Balanophora fungosa* on melanin. The 50% EtOH extract obtained from *B. fungosa* indicated an inhibitory effect on mushroom tyrosinase activity with an IC_{50} value of 15 μg/mL. Bioassay-guided fractionation of the active extract resulted in the isolation of four known compounds. Their structures were identified as 1-*O*-(*E*)-caffeoyl-3-*O*-galloyl-4,6-(*S*)-HHDP-β-D-glucopyranose (**1**), 1-*O*-(*E*)-caffeoyl-3,4,6-tri-*O*-galloyl-β-D-glucopyranose (**2**), caffeoyl-β-D-glucopyranose (**3**), and abietin (**4**) on the basis of spectroscopic analyses and comparison of their spectral data with those in the literature. Compounds **1** and **2** prevented pigmentation of melanin in a three-dimensional cultured human skin model. Furthermore, compounds **1** and **2** indicated inhibitory activities against trypsin and tryptase.

KEYWORDS: *Balanophora fungosa*, melanin, three-dimensional cultured human skin model, tyrosinase activity, trypsin activity, tryptase activity

■ INTRODUCTION

Dendritic cells known as melanocytes, which are located at basal lamina, are responsible for producing melanin in the skin.[1] Tyrosinase (EC 1.14.18.1) synthesized in the melanocytes plays an important role in melanin synthesis, such as the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA), oxidation of L-DOPA to dopaquinone, and oxidation of 5,6-dihydroxyindole to indolequinone.[2−6] Keratinocytes ingest the synthesized melanin through the melanocyte dendrite, following an increase of protease-activated receptor 2 (PAR-2) activation.[7−11] Trypsin and mast cell tryptase are known to be activators of PAR-2.[12,13] Accordingly, the inhibitors of these enzymes (tyrosinase, trypsin, and tryptase) have the potential to become hypopigment agents, which prevent the overproduction of melanin in the skin.[14−16]

In our ongoing research for bioactive substances from Okinawan plants,[17] we investigated *Balanophora fungosa* J.R. et G. Forst. (syn. *B. kuroiwai* Makino), which is a parasitic plant growing on the root of various plants such as *Pongamia pinnata*, *Macaranga tanarius*, and *Diospyros maritima*.[18] We discovered that a 50% EtOH extract of *B. fungosa* indicated potent inhibition of tyrosinase, trypsin, and tryptase activities and also showed hypopigment activity in a three-dimensional human skin tissue. Although various compounds, such as steroids, lignans, galloyl and hexahydroxydiphenoyl esters of phenylpropanoid glucosides, and ellagitannins, have been reported from *B. fungosa*,[19] there is yet no report about its hypopigmentation effect. This paper deals with the inhibitory effect of melanin synthesis by *B. fungosa*.

■ MATERIALS AND METHODS

Materials. *B. fungosa* was collected from Ishigaki-jima, Okinawa, Japan. Extraction of sample was performed on an ASE 200 accelerated solvent extractor (Dionex Corp., Sunnyvale, CA). Briefly, 2 g of air-dried sample was mixed with 8 g of Celite. The mixture was transferred to a 33 mL extraction cell and extracted with H_2O/EtOH (1:1, 50 mL) to yield 50% EtOH extract (1.3 g). The three-dimensional (3D) cultured human skin models [MelanoDerm (MEL-300-B), normal human epidermal melanocytes (NHEM) from dark skin donors, and normal human epidermal keratinocytes (NHEK), cocultured on the surface of a collagen-coated membrane to generate several cell layers] were purchased from Kurabo Industries Ltd. (Osaka, Japan). The human skin models were maintained using long life maintenance medium (EPI-100LLMM, which consisted of bFGF and α-MSH, Kurabo Industries Ltd.) at 37 °C in a humidified atmosphere that contained 5% CO_2, according to the manufacturer's instructions. Human melanoma cells, HMV-II, were provided by BioResource Center, RIKEN, Japan. Proteinase K, mushroom tyrosinase, L-DOPA, heparin, and L-BAPA were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Human lung tryptase and porcine pancreatic trypsin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM λ 400 FT-NMR spectrometer, and chemical shifts were referenced to the solvent signals δ_H 2.49 and δ_C 39.5 in DMSO-d_6. Inversed-detected heteronuclear correlations were measured using HMQC and HMBC pulse sequences with a pulse field gradient. LR-ESIMS data were measured on a Waters Quattro micro API triple-quadruple mass analyzer. Preparative RP-HPLC was run on a Waters 600 multisolvent system using ODS columns (YMC-Pack ODS-A, 2 cm i.d. × 25 cm, YMC-Pack ODS-AQ, 2 cm i.d. × 25 cm). YMC-ODS AM was used for chromatography, 5 cm i.d. × 30 cm. All solvents used were of reagent grade.

Enzymatic Assay of Tyrosinase. Tyrosinase activity using L-DOPA as the substrate was evaluated on the basis of the method of Likhitwitayawuid et al. [20] with slight modification. To a 96-well plate were added 20 μL of each sample solution (dissolved in 5% DMSO), 40 μL of mushroom tyrosinase (40 U/mL, Sigma-Aldrich Corp.), and 100 μL of 67 mM phosphate buffer (pH 6.8). After preincubation at 23 °C for 3 min, 50 μL of 2.5 mM L-DOPA was added in the 96-well plate, and the optical density at 490 nm of each well was measured using a microplate reader (model 550, Bio-Rad Laboratories, Inc., Richmond, CA). After incubation at 23 °C for 10 min, the increase in the optical density at 490 nm was measured. The extent of inhibition upon addition of sample is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC$_{50}$). The percentage inhibition of tyrosinase activity was calculated by the following equation: % inhibition (%) = $[1 - (A - B)/C] \times 100$, where A is the optical density at 490 nm with test sample and enzyme, B is the optical density at 490 nm with the test sample and without enzyme, and C is the optical density at 490 nm with enzyme without test sample.

Culture for the Human Skin Model. The 3D human skin model was placed on a 6-well plate with 0.9 mL of medium and incubated at 37 °C for 1 h in a humidified atmosphere, which contained 5% CO$_2$ in air. After incubation, the skin model was placed on another 6-well plate that contained 5 mL of fresh medium. An aliquot (0.1 mL) of both test samples (0−2 mg/mL) of *B. fungosa* extract dissolved in 12.5% glycerol and that of positive control (1 mg/mL of arbutin) were applied on the surface of the tissue on model cup ($n = 3$). The tissue was cultured for 10 days and refed with 0.1 mL of the sample solution and fresh LLMM every 2 days.

At the end of culture, the melanin production and the viability of the tissue cells were measured.

Melanin Quantitation for the Human Skin Model. The melanin production of the tissue cells of the 3D human skin model was measured using a minor modification to the method of Bessou-Touya et al.[9] At the end of culture, the skin model was placed on a 24-well plate, and the tissue surface was rinsed three times with Dulbecco-PBS. The tissue was treated with 0.45 mL of 10 mM Tris-HCl buffer (pH 6.8, which contained 1% SDS and 0.05 mM EDTA) and incubated at room temperature for 3 h in an airtight container with 20 μL of 5 mg/mL proteinase K. Following the incubation, the tissue was transferred from the model cup to a 1.5 mL tube and again incubated at 45 °C overnight. To remove coloring matter, the tube was centrifuged at 20000g for 15 min, and the supernatant was discarded. The precipitated tissue was washed with 0.45 mL of 10 mM Tris-HCl (pH 6.8, which contained 0.05 mM EDTA), and the supernatant was removed after centrifugation. The washed tissue was reacted with 20 μL of 5 mg/mL proteinase K and 0.45 mL of 10 mM Tris-HCl (pH 6.8, which consisted of 1% SDS and 0.05 mM EDTA). The obtained lysate was mixed with 0.5 M sodium carbonate (50 μL) and 30% hydrogen peroxide (10 μL), incubated at 80 °C for 30 min, and then allowed to cool. The lipid from the tissue was removed by the addition of 100 μL of a chloroform/methanol (2:1) mixture followed by centrifugation at 10000g for 10 min. The optical density of the aqueous phase was measured at 405 nm on a 96-well plate with a microplate reader (model 680, Bio-Rad).

Cell Viability of the Human Skin Model. The viability of the tissue cells of the 3D human skin models was determined with the MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] dye reduction assay. At the end of culture, the model cup was rinsed three times with Dulbecco-PBS. The skin model was placed on a 24-well plate, 300 μL of MTT solution (MTT-100 kit, Kurabo) was added, and the tissue was incubated at 37 °C for 3 h in a humidified atmosphere, which contained 5% CO$_2$. After incubation, the tissue was washed with Dulbecco-PBS. The skin model was placed on another 24-well plate, 2 mL of MTT extraction solution (provided with the kit) was added to each well, and the plate was shaken at room temperature for 2 h. The

optical density of 200 μL of the extract was measured at 570 nm on a 96-well plate with a microplate reader (model 680).

Assay of Melanin Content in HMV-II Melanoma Cells. Human melanoma cells (HMV-II) were cultured in Ham's F12 medium supplemented with 20% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 ng/mL streptomycin. The inhibitory effect of the isolated compounds was examined as follows. HMV-II cells were seeded at a density of 7.5×10^4 cells/well in 1 mL of medium on a 24-well plate and incubated at 37 °C for 1 day in a humidified atmosphere, which contained 5% CO$_2$. After incubation, the culture medium was changed to 995 μL of fresh one, and 5 μL of the test sample solution (dissolved in DMSO) was supplied to the well ($n = 3$). Five microliters of 80 mM arbutin (dissolved in DMSO) was used as positive control. The cells were cultured for 11 days. On days 5 and 8, the medium was changed to fresh medium containing various concentrations of test sample.

At the end of culture, the melanin production was measured. The cultured cells were solubilized by treatment with 200 μL of 1 N NaOH aqueous solution at room temperature overnight. The lysate (190 μL) was transferred to a 96-well flat-bottom microplate, and the absorbance of these solutions was measured at 405 nm with a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc.). A growth inhibition experiment was carried out in parallel, and the amount of viable cells at the end of culture was determined with the MTT dye reduction assay. One hundred microliters of 5 mg/mL MTT in PBS was added to each well containing 1 mL of medium, and the plate was again incubated. After 3 h of standing, the medium was removed, and the resulting formazan crystals were dissolved with 1 mL of DMSO. The optical density of 200 μL of the extract was measured at 570 nm, providing the reference for reading at 620 nm on a 96-well plate with a microplate reader (Multiskan FC).

Enzymatic Assay of Serine Protease. Serine protease activities were evaluated by two methods: porcine pancreatic trypsin and human lung tryptase. Trypsin activity using BAPA as the substrate was assayed on the basis of the method of Lottenberg et al.[21] To a 96-well plate were added 10 μL of sample solution (dissolved in water) and 50 μL of 30 μg/mL trypsin [dissolved in 50 mM Tris-HCl buffer (pH 8.0, containing 10 mM CaCl$_2$)], and the plate was shaken at 25 °C for 5 min. The substrate of 100 μL of 0.75 mM BAPA [dissolved in 50 mM Tris-HCl buffer (pH 8.0, which consisted of 10 mM CaCl$_2$ and 1% DMSO)] was added, and the optical density at 405 nm of each well was measured using a microplate reader (model 680). After 20 min of shaking at 25 °C, the increase in the optical density at 405 nm of each well was measured using a microplate reader. The extent of inhibition upon addition of sample is expressed as the concentration at which 50% of the trypsin activity was inhibited (IC$_{50}$). Tryptase activity using BAPA as the substrate was assayed spectrophotometrically with minor modification.[22] An aliquot of 10 μL of sample solution (dissolved in water) and 50 μL of 3.3 μg of protein/mL tryptase [dissolved in 40 mM Tris-HCl buffer (pH 8.1, which consisted of 0.15 M NaCl, 10 mM CaCl$_2$, and 77 μg/mL heparin] were added to a 96-well plate. After preincubation at 25 °C for 5 min, 100 μL of 0.75 mM BAPA [dissolved in 40 mM Tris-HCl buffer (pH 8.1, which contained 0.15 M NaCl, 10 mM CaCl$_2$, 77 μg/mL heparin and 1% DMSO] was added to each well, and the optical density at 405 nm was measured using a microplate reader (model 680). After incubation at 25 °C for 30 min, the increase in the optical density at 405 nm was measured. The extent of inhibition upon addition of sample is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC$_{50}$).

Extraction and Isolation of Compounds 1−4 . The ground part (peduncle) of *B. fungosa* was lyophilized and crushed to powder. An aliquot of the lyophilized powder (100 g) was extracted with H$_2$O/EtOH (1:1, 500 mL × 2) and filtered to remove debris. The filtrate was extracted with CH$_2$Cl$_2$ (350 mL) to yield crude CH$_2$Cl$_2$-soluble material (5.0 g). The resultant aqueous phase was concentrated in vacuo to remove EtOH and then partitioned between *n*-BuOH (350 mL) and H$_2$O (500 mL) to yield *n*-BuOH-soluble (28.3 g) and H$_2$O-soluble (37.7 g)
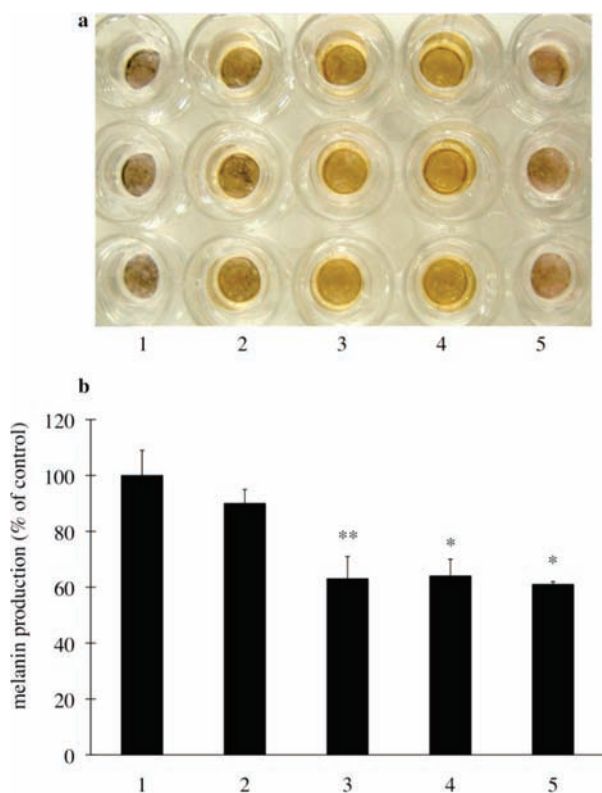
Figure 1. Extract of *B. fungosa* prevented pigmentation of melanin in a three-dimensional cultured human skin model: (a) macroscopic view of cultured tissues; (b) melanin of the tissues was quantitated. Various concentrations of the extract were applied: 1, control; 2, 0.5 mg/mL; 3, 1 mg/mL; 4, 2 mg/mL; 5, 1 mg/mL of arbutin as positive control. Bars represent the mean $\pm$ SD ($n = 3$). $*$, significantly different from the control at $p < 0.05$; $**$, significantly different from the control at $p < 0.01$.

materials, respectively. The *n*-BuOH-soluble material (28.3 g) was separated by chromatography on ODS [YMC-ODS AM, 5 cm i.d. $\times$ 30 cm, linear gradient elution, H_2O/MeOH (7:3)$-$MeOH] to give five fractions designated F_1-F_5, and then fraction F_3 (6.2 g) was fractioned by HPLC [YMC ODS-A, 2 cm i.d. $\times$ 25 cm, H_2O/MeOH (3:2)] to give five fractions, $F_{3-1}-F_{3-5}$. HPLC purification of the third fraction (F_{3-3}, 1.1 g) yielded **1** (180 mg). An aliquot of 0.75 g of fraction F_{3-4} (6.0 g) was separated by HPLC [YMC ODS-AQ, 2 cm i.d. $\times$ 25 cm, 1% formic acid/H_2O/MeOH (2:11:7)] to give **2** (124 mg). An aliquot of 0.31 g of fraction F_1 (6.1 g) was fractionated by HPLC [YMC ODS-A, 2 cm i.d. $\times$ 15 cm, H_2O/MeCN (19:1)] and then purified by HPLC [YMC ODS-A, 2 cm i.d. $\times$ 15 cm, linear gradient elution, 1% formic acid/H_2O/MeCN (1:9:0)$-$(1:8:1)] to afford **3** (14.2 mg). Compound **4** was isolated from another separation step of *B. fungosa* extract as follows. The lyophilized powder (12 g) of the ground part of *B. fungosa* was extracted with H_2O/EtOH (1:1, 250 mL), and the extract was separated by open column chromatography on Diaion HP20 eluted with H_2O/EtOH (19:1; 3 L, 3:1; 1.5 L) and H_2O/EtOH (1:1; 1.5 L), successively. The H_2O/EtOH (1:1) part (1.44 g) contained **1** and **2**. Purification of the H_2O/EtOH (3:1) part (704 mg) by a series of HPLC [YMC ODS-A, 2 cm i.d. $\times$ 15 cm, linear gradient elution, H_2O/MeOH (4:1)$-$(7:3)] and [YMC ODS-A, 2 cm i.d. $\times$ 15 cm, linear gradient elution, H_2O/MeOH (17:3)$-$(7:3)] led to the isolation of **3** (0.6 mg) and **4** (4.2 mg).

■ RESULTS AND DISCUSSION

Effect of *B. fungosa* Extract on Melanin Synthesis in a 3D Cultured Human Skin Model. To determine the inhibitory

Table 1. Inhibitory Activities of Various Fractions Obtained from the Extract of *B. fungosa* on Mushroom Tyrosinase Activity

	IC_{50} value^a (μg/mL)
B. fungosa extract	15
CH_2Cl_2 material	8.2
BuOH material	9.6
H_2O material	360

^{a} Inhibitory activity was expressed as the mean of 50% inhibitory concentrations in quintuplicates, obtained by interpolation of concentration inhibition curve.
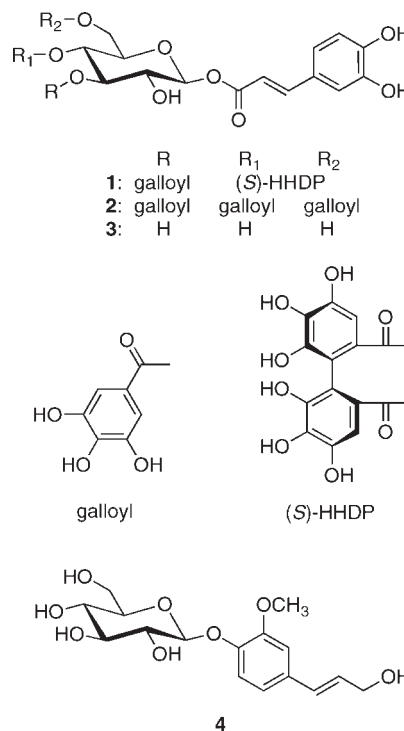

Figure 2. Structures of compounds **1**$-$**4**.

effect of *B. fungosa* extract on melanogenesis, we cultured a 3D human skin model with various concentration of the extract for 10 days. As a result, the extract and arbutin showed clearly inhibited pigmentation compared with control (Figure 1a), as quantified in Figure 1b.

To exclude the possibility that the above inhibitory effects of the *B. fungosa* extract on melanogenesis might have been caused by the inhibition of cell growth, we evaluated the cell viability of the 3D human skin model that has been topically applied with and without the extract by MTT assay. No significant cytotoxicity was observed when 4 mg/mL of the extract was applied on the tissue (4 mg/mL of the extract, 107 $\pm$ 4%; 1 mg/mL of arbutin, 98 $\pm$ 7%). Therefore, the extract of *B. fungosa* might have decreased melanin pigmentation without affecting the cell viability.

Identification and Activities of Compounds 1$-$4. The extract of *B. fungosa* inhibited the oxidation of L-DOPA (catalyzed by tyrosinase) with an IC_{50} value of 15 μg/mL (Table 1). To identify the active metabolites, we evaluated the solvent-soluble fractions including CH_2Cl_2, and *n*-BuOH, as well as the H_2O layer derived from the 50% EtOH extract of *B. fungosa*. As summarized in Table 1, some solvent-partitioned

Table 2. Inhibitory Activities of Compounds 1−4, Obtained from *B. fungosa*, on Tyrosinase, Tryptase, and Tryptase Activities

| | IC$_{50}$ value^a (μg/mL) | | |
compd	tyrosinase activity	trypsin activity	tryptase activity
1	5.7	12	2.2
2	5.9	7.9	1.0
3	>25	nd	>125
4	>100	nd	>125
positive controls^b	5.0	0.10	0.11

^{a} Inhibitory activity was expressed as the mean of 50% inhibitory concentrations, obtained by interpolation of concentration inhibition curve. nd, not done. ^{b} Kojic acid used in tyrosinase assay and leupeptin in trypsin and tryptase assays.
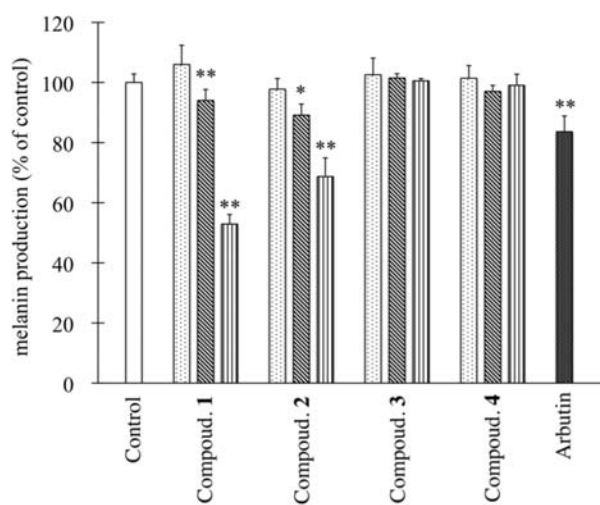

Figure 3. Compounds **1** and **2** prevented melanin production in HMV-II human melanoma cells; 0.4 mM arbutin was used as positive control. The melanin of the cells was quantitated. Bars represent the mean ± SD ($n = 3$). *, significantly different from the control at $p < 0.05$; **, significantly different from the control at $p < 0.01$.

fractions such as CH$_2$Cl$_2$- and *n*-BuOH-soluble fractions inhibited the oxidation of L-DOPA (catalyzed by tyrosinase) with IC$_{50}$ values of 8.2 and 9.6 μg/mL, respectively. Tyrosinase assay-guided fractionation of the *n*-BuOH-soluble material led to the isolation of tyrosinase inhibitory metabolites **1** and **2** and inactive compound **3**. Compound **4** was also isolated from another separation step of *B. fungosa* extract. These metabolites were identified as 1-*O*-(*E*)-caffeoyl-3-*O*-galloyl-4,6-(*S*)-HHDP-β-D-glucopyranose (**1**), 1-*O*-(*E*)-caffeoyl-3,4,6-tri-*O*-galloyl-β-D-glucopyranose (**2**), caffeoyl-β-D-glucopyranose (**3**), and abietin (**4**) on the bases of spectral analyses and comparison of their spectral data with those in the literature as previously reported.[19,23] The structures of the four metabolites (**1−4**) are presented in Figure 2. IC$_{50}$ values of compounds **1** and **2** on the oxidation of L-DOPA (catalyzed by tyrosinase) were determined to be 5.7 and 5.9 μg/mL, respectively, as shown in Table 2. Compounds **3** and **4** showed weak inhibition against tyrosinase activity, with 40% inhibition at 25 μg/mL and 18% inhibition at 100 μg/mL, respectively (Table 2). The values were compared with kojic acid, as positive control, with an IC$_{50}$ value of 5.0 μg/mL.

Table 3. Cytotoxicity of Compounds 1−4 in HMV-II Human Melanoma Cells^a

| | % of control (mean) ± standard deviation | |
compd	12.5 μg/mL	25 μg/mL
1	119 ± 8	50 ± 7
2	98 ± 8	100 ± 6
3	91 ± 8	97 ± 6
4	105 ± 4	104 ± 3

^{a} Cells were cultured for 11 days with various concentrations of test compounds in quadruplicates.
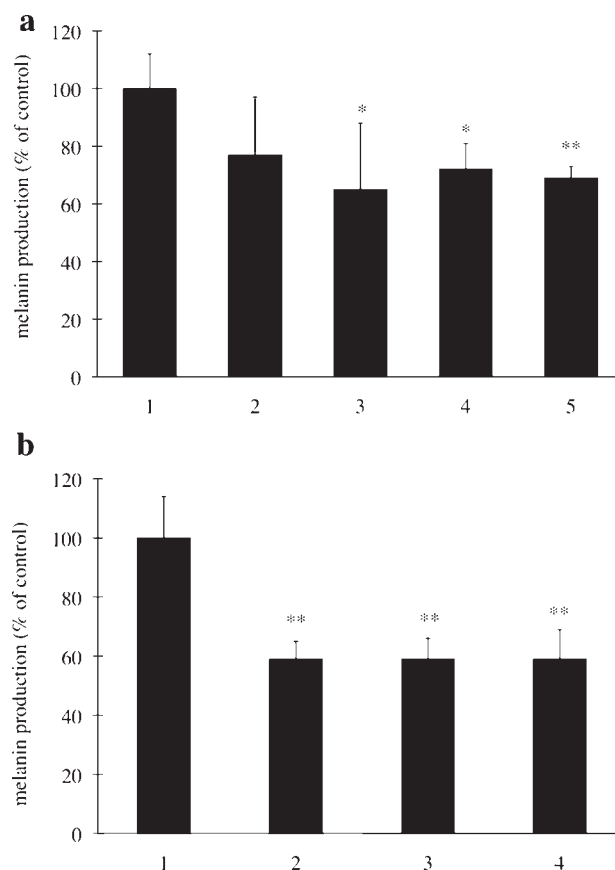

Figure 4. Compounds **1** and **2** prevented pigmentation of melanin in a three-dimensional cultured human skin model: (a) various concentrations of **1** were applied (1, control; 2, 0.5 mg/mL; 3, 1 mg/mL; 4, 2 mg/mL; 5, 1 mg/mL of arbutin as positive control); (b) various concentrations of **2** were applied (1, control; 2, 0.5 mg/mL; 3, 1 mg/mL; 4, 1 mg/mL of arbutin as positive control). The tissue was cultured for 14 days and refed with 0.1 mL of the sample solution and fresh LLMM every 3 days. At the end of culture, melanin production was quantitated. Bars represent the mean ± SD ($n = 4$). *, significantly different from the control at $p < 0.05$; **, significantly different from the control at $p < 0.01$.

To investigate the effect of compounds **1−4** on melanogenesis, we cultured human melanoma cells (HMV-II) with various concentrations of **1−4** for 11 days. As shown in Figure 3, at concentrations of 12.5 μg/mL of **1** and 12.5 and 25 μg/mL of **2**, melanin accumulation was reduced to 94, 89, and 69% of the control, respectively. However, compounds **3** and **4** did not inhibit melanin synthesis on HMV-II (Figure 3). To exclude the possibility that the inhibitory effects of compounds **1** and **2** on
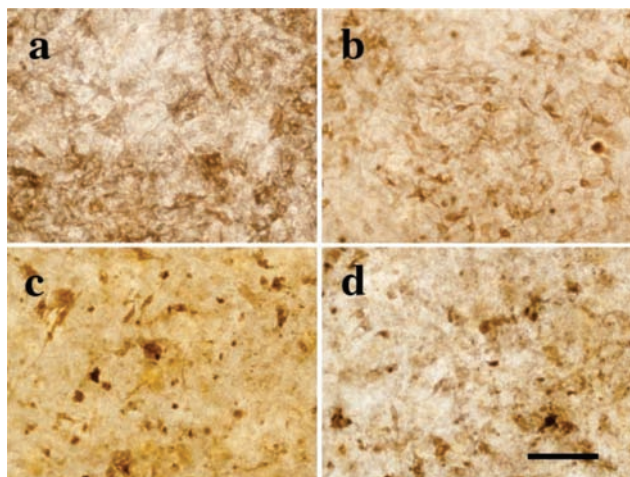
Figure 5. Microscopic views of a three-dimensional cultured human skin model: (a) control; (b) 1 mg/mL of arbutin as positive control; (c) 2 mg/mL of **1**; (d) 2 mg/mL of **2**. Dark cells are melanocytes in tissue. Scale bar = 200 μm. The tissue was cultured for 13 days and refed with 0.1 mL of the sample solution and fresh LLMM every 3 days.

Table 4. Cytotoxicity of Compounds 1 and 2 on a Three-Dimensional Human Skin Model[a]

compd	% of control (mean) $\pm$ standard deviation	
	1 mg/mL	2 mg/mL
1	97 ± 7^b	85 ± 8^c
2	nd^e	98 ± 10^d

[a] Tissues were cultured for 13 days with various concentrations of test compounds. [b] $n = 5$. [c] $n = 3$. [d] $n = 3$. [e] nd, not done.

melanogenesis might be caused by the inhibition of cell growth, we evaluated the cell viability of compounds **1** and **2** by MTT assay. As shown in Table 3, 12.5 μg/mL of compound **1** and 12.5 and 25 μg/mL of compound **2** did not show cytotoxicity against HMV-II cells. However, the number of cells was reduced to 50% at 25 μg/mL of compound **1**. These results indicate that compounds **1** and **2** had inhibitory effects on melanogenesis at noncytotoxic concentrations.

To verify the inhibitory activity of melanogenesis with compounds **1** and **2**, we cultured a 3D human skin model with various concentrations of **1** and **2** for 14 days. As a result, compounds **1** and **2** showed clearly inhibited pigmentation compared with control (Figure 4). When the culture tissue was observed under an inverse microscope, melanin appeared to spread around melanocytes on control and arbutin-applied tissue (Figure 5a,b). On the other hand, melanin appeared to be localized on melanocyte in the compound-applied tissue (Figure 5c,d). From this localization, we assumed that the extract might have inhibited melanosome transfer from melanocytes to keratinocytes. No significant cytotoxicity was observed when 2 mg/mL of each compound was applied on the tissue, as shown in Table 4.

We also found that the extract of *B. fungosa* had inhibitory activities against trypsin and tryptase with IC$_{50}$ values of 75 and 22 μg/mL, respectively. We then evaluated the inhibitory effect of these compounds. Compounds **1** and **2** showed inhibition of trypsin with IC$_{50}$ values of 12 and 7.0 μg/mL, respectively. Futhermore, compounds **1** and **2** showed inhibition of tryptase with IC$_{50}$ values of 2.2 and 1.0 μg/mL, respectively, as shown in

Table 2. Compounds **3** and **4** showed no inhibition of typtase activity at 125 μg/mL. In contrast to leupeptin as positive control, the compounds indicated more selective inhibition against tryptase activity than trypsin activity. We assumed that the melanin localization observed in 3D human skin tissues was triggered by inhibition of serine protease activity of compounds **1** and **2**.

This is the first study demonstrating an inhibitory effect of *B. fungosa* on melanogenesis. The 50% EtOH extract of *B. fungosa* inhibited melanin synthesis in a 3D human skin model and tyrosinase, trypsin, and tryptase activities. The ground part of *B. fungosa* contains compounds **1** and **2** with concentration as high as 0.18 and 0.99%, respectively. We assume that compounds **1** and **2** are the major components of melanin synthesis inhibitors of *B. fungosa*. Compounds **1** and **2** indicated inhibitory activity against tyrosinase with IC$_{50}$ values of 5.7 and 5.9 μg/mL, respectively. However, compound **3** did not inhibit tyrosinase activity. These results indicate that galloyl groups at C-3 in compound **1** and at C-3, C-4 and C-5 in compound **2** and/or a HHDP group at C-4,5 in compound **1** are important factors for inhibitory activity against tyrosinase.

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