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Melanogenesis inhibitors from the rhizomes of *Alpinia officinarum* in B16 melanoma cells

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ABSTRACT

The 80% aqueous acetone extract from the rhizomes of *Alpinia officinarum*, a Chinese medicinal herb, were found to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells. Among the constituents isolated, four diarylheptanoids [5-hydroxy-1,7-diphenyl-3-heptanone, 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one, 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone, and 3,5-dihydroxy-1,7-diphenylheptane] and two flavonol constituents (kaempferide and galangin) inhibited melanogenesis with IC_{50} values of 10–48 μM, and several structural requirements of the active constituents for the inhibition were clarified. In addition, 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one, kaempferide, and galangin inhibited mRNA expression of tyrosinase and tyrosinase-related proteins-1 and -2, and the protein level of a microphthalmia-associated transcription factor.

1. Introduction

Melanin production is principally responsible for skin color, and melanin pigmentation is a major defense mechanism against ultraviolet rays from the sun. Melanin biosynthesis proceeds through a complex series of enzymatic and chemical reactions in melanocytes.[1–4] The synthesis of melanin starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, the enzyme catalyzing the rate-limiting step of melanin biosynthesis.[2] This tyrosinase process is involved in abnormal accumulation of melanin pigments (hyperpigmentation).[5,6] Therefore, tyrosinase inhibitors such as kojic acid and albutin have been established as important constituents of cosmetic products and depigmenting agents for the treatment of hyperpigmentation.[7,8]

In the course of our studies on the bioactive constituents of natural medicines, we found that the 80% acetone extract from the dried rhizomes of *Alpinia officinarum* HANCE showed inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. The Zingiberaceae plant *A. officinarum* has been cultivated in southern China and Taiwan, and the rhizomes of this plant are used as a spice and as a traditional medicine for several purposes, such as a stomachic and carminative, in China. In chemical studies of these rhizomes, the isolation and structural determinations of monoterpenes,[9] diarylheptanoids,[10–16] flavonoids,[17–19] phenylpropanoids,[20,21] and neolignans[22] have been reported. With regard to the biological effects, antiemetic,[16] antioxidant,[21,22] and anti-genotoxic activities[23] and inhibition of prostaglandin and leukotriene biosynthesis,[14,24] pancreatic lipase,[19,25] 5α-reductase,[26] and of nitric oxide production,[27–29] etc. were reported. Lu et al. reported that the extract of *A. officinarum* and galangin (**6**) inhibited the enzyme activity of mushroom tyrosinase and melanogenesis in B16 melanoma cells.[30] However, the effects of other constituents of this natural medicine on melanogenesis in B16 melanoma cells have not been reported to the best of our knowledge.

In this paper, we describe the effects of constituents (**1**–**9**) from the rhizomes of *A. officinarum*[29] and related flavonoids (**10**, **11**)[31,32] on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells and on the enzyme activity of mushroom tyrosinase.

2. Results and discussion

2.1. Effects of constituents (1–9) and related flavonoids (10, 11) on melanogenesis

Melanocytes can be stimulated by many effectors including ultraviolet radiation[33] and α-melanocyte-stimulating hormone (α-MSH).[34] It is generally accepted that the cAMP pathway plays a key role in the regulation of melanogenesis, and cAMP is involved in α-MSH-stimulated signal transduction.[34,35] In the present study, we used the phosphodiesterase inhibitor theophylline[36] to stimulate B16 melanoma 4A5 cells. As shown in Table 1, the 80% acetone extract significantly inhibited melanogenesis with an IC_{50} value of

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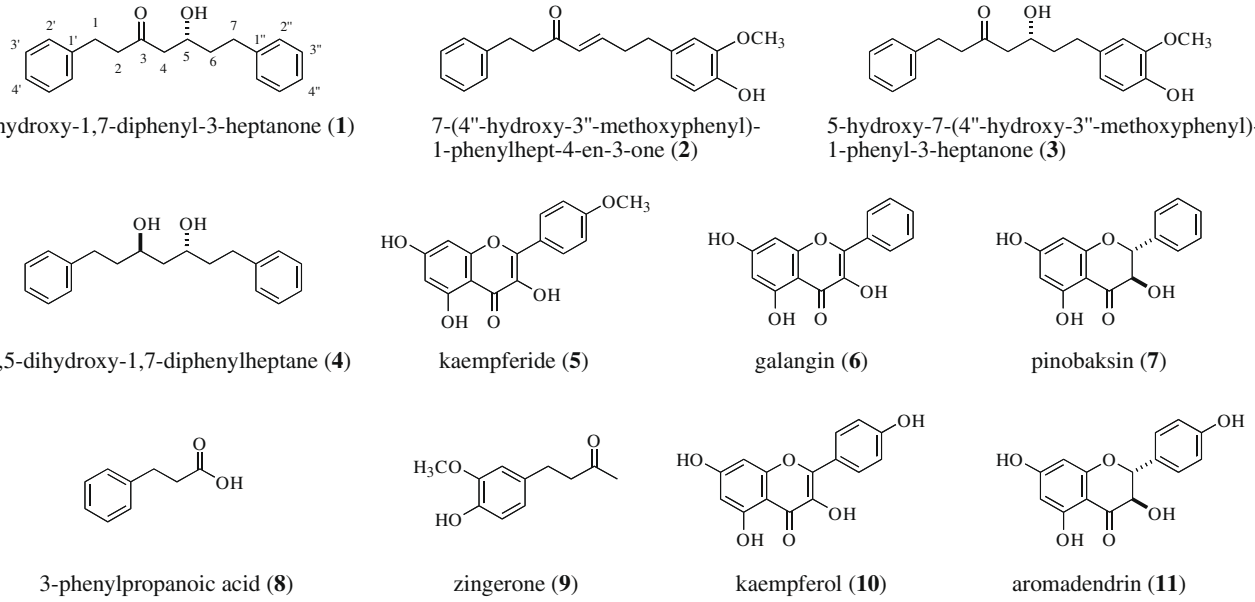

Figure 1. Chemical structures of compounds (**1**–**9**) isolated from the rhizomes of *A. officinarum* and related flavonoids (**10**, **11**).

44 μg/mL. The extract was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble fraction and water-soluble fraction. The EtOAc-soluble fraction, which showed potent activity (IC_{50} value = 8.1 μg/mL), was subjected to column chromatographies and finally HPLC to give **1**–**9** (Fig. 1).[29]

Among the constituents isolated, a diarylheptanoid [7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**2**)] and flavonols [kaempferide (**5**), galangin (**6**)] substantially inhibited melanogenesis with IC_{50} values of 10 μM, 16 μM, and 17 μM, respectively (Fig. 2, Table 2). 5-Hydroxy-1,7-diphenyl-3-heptanone (**1**), 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**3**), and 3,5-dihydroxy-1,7-diphenylheptane (**4**) also showed moderate activity (IC_{50} values = 30–48 μM). These active compounds (**1**–**6**) showed greater activity than that of arbutin.

Two flavonols, kaempferide (**5**) and galangin (**6**), inhibited melanogenesis in the cells. However, pinobanksin (**7**) did not show such effects. In addition, a related flavonol, kaempferol (**10**), also inhibited melanogenesis (IC_{50} value = 20 μM), but the corresponding dihydroflavonol, aromadendrin (**11**), showed less inhibition (IC_{50} value = 84 μM) (Table 2). In addition, cytotoxic effects were observed after incubation with **2**, **4**, **6**, and **10** at a high concentration (100 μM) (Table 3).

With regard to the inhibitory effects of diarylheptanoids on the melanogenesis of B16 melanoma cells, several diarylheptanoids, such as (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-glucoside, (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-ol, oregonin, and hirsutanonol from *Alnus hirsute*,[37] and aceroside I and acerogenin M from *Acer nikoense*[38] were reported to be active principles. In the present study, compound **2** showed the greatest activity among the four diarylheptanoids, suggesting that an enone moiety at the 3–5 positions is important for greater activity [**2** (IC_{50} value = 10 μM) >**3** (30 μM)].

On the other hand, flavonoids that inhibit melanogenesis and those that conversely enhance it were reported. For example, luteolin inhibits tyrosinase activity and melanin production by inhibiting adenyl cyclase in α-MSH-stimulated B16 melanoma cells.[39]

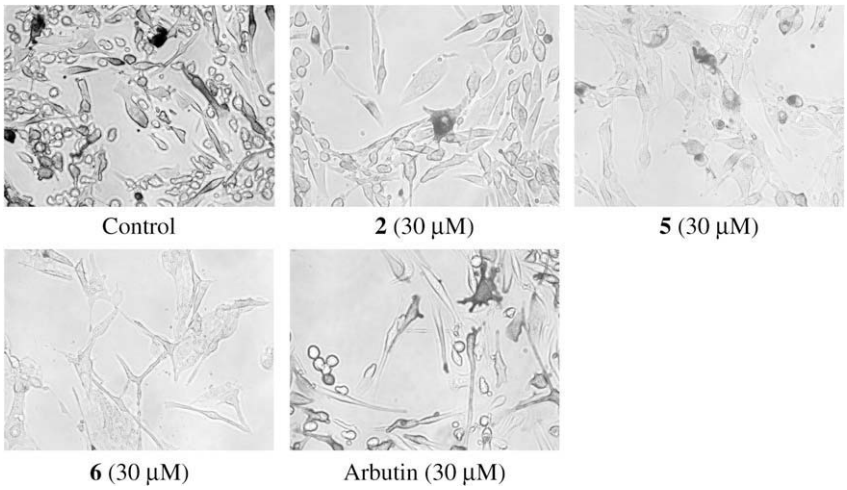

Figure 2. Photographs of theophylline-stimulated B16 melanoma 4A5 cells 72 h after treatment with **2**, **5**, **6**, and arbutin. The melanoma cells (1.6×10^4 cells/ 400 μL/well) were seeded into 24-well multiplate. After 24 h of culture, the test compound and theophylline 1 mM were added and incubated for 72 h. The images are representative of several experiments.

Table 1
Inhibitory effects of 80% acetone extract and EtOAc- and H_2O-soluble fractions from *Alpinia officinarum* on melanogenesis and proliferation in B16 melanoma 4A5 cells

	Concentration (μg/mL)						
	0	1	3	10	30	100	IC_{50} (μg/mL)
Inhibition of melanogenesis (%)							
80% Acetone extract	0.0 ± 5.6	2.0 ± 6.0	6.8 ± 6.7	12.3 ± 4.0	36.4 ± 2.1**	69.9 ± 5.5**	44
EtOAc-soluble fraction	0.0 ± 4.5	4.4 ± 2.6	26.0 ± 2.0**	53.8 ± 2.4**	88.4 ± 1.3**	–	8.1
H_2O-soluble fraction	0.0 ± 5.9	17.1 ± 3.7*	15.8 ± 3.4*	14.9 ± 3.1	12. 2 ± 3.9	7.4 ± 1.9	–
Inhibition of proliferation (%)							
80% Acetone extract	0.0 ± 1.2	−3.7 ± 1.8	−2.8 ± 0.7	4.8 ± 0.7	7.9 ± 1.0**	10.4 ± 3.5**	–
EtOAc-soluble fraction	0.0 ± 0.5	3.5 ± 0.6	5.8 ± 0.6*	11.5 ± 0.8**	44.0 ± 1.2**	99.8 ± 0.1**	29
H_2O-soluble fraction	0.0 ± 1.8	−1.4 ± 1.6	−1.9 ± 0.6	−2.5 ± 0.4	1.0 ± 0.9	−4.9 ± 1.5	–

Each value represents mean ± S.E.M. (n = 4).
Significantly different from the control.
* p <0.05.
** p <0.01.

Pedalitin, quercetin, and nobiletin were reported not to suppress the production of melanin but rather to enhance it.[40–42]

In the present study, two flavonols, kaempferide (**5**) and galangin (**6**), inhibited melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. However, pinobanksin (**7**) did not show such effects. In addition, a related flavonol, kaempferol (**10**), also inhibited melanogenesis, but the corresponding flavanonol aromadendrin (**11**) showed less activity. These findings suggest that the 2,3-double bond of the active flavonols (**5**, **6**, **10**) is essential for the greater melanogenesis-inhibitory activity.

2.2. Effects on mushroom tyrosinase

Mushroom tyrosinase has conventionally been used for the development of tyrosinase inhibitors.[8] In agreement with the previous reports,[30,43] galangin (**6**) and kaempferol (**10**) showed moderate inhibitory activity under our experimental conditions [inhibition (%): 34.4% and 38.4%, respectively, at a concentration of 100 μM], but other inhibitors (**1–5,11**) of melanogenesis showed only very weak inhibition (~21.0%) (Table 4).

2.3. Effects on mRNA expression of tyrosinase and TRP-1 and -2

The tyrosinase-related protein (TRP) family including tyrosinase, TRP-1, and TRP-2 are known to be enzymes catalyzing the major steps in melanin synthesis.[44] Galangin (**6**) was reported to inhibit melanogenesis in B16 melanoma cells and mushroom tyrosinase activity,[30] but the effects of **6** on the mRNA expression of tyrosinase and TRP-1 and -2 were not clarified. Therefore, we examined the effects of **2** and **5** as well as **6** on the mRNA expression of tyrosinase and TRP-1 and -2 in the B16 melanoma 4A5 cells and found that all three compounds inhibited mRNA expression of tyrosinase and TRP-1 and -2 in a concentration-dependent manner (Fig. 3).

2.4. Effects on protein levels of MITF

Recently, microphthalmia-associated transcriptional factor (MITF), has been shown to play a key role in melanocyte survival, development, and differentiation and to be a target for the inhibition of melanogenesis.[45–48] Therefore, protein levels of MITF after incubation with **2**, **5**, and **6** were examined using Western blotting. After incubation with **2**, **5**, and **6** for 24 h, weak inhibition of MITF protein levels was observed (data not shown), but incubation with those compounds for 48 h markedly reduced the protein levels of MITF (Fig. 4). These findings suggest that **2**, **5**, and **6** inhibit melanogenesis in theophylline-stimulated B16-4A5 melanoma cells by inhibiting MITF expression, at least in part.

3. Conclusion

The 80% acetone extract of the rhizomes of *A. officinarum* were found to inhibit melanogenesis in theophylline-stimulated mouse B16 melanoa 4A5 cells. Among the constituents isolated, four

Table 2
Effects of constituents (**1**–**9**) isolated from *Alpinia officinarum* and related flavonoids (**10**, **11**) on melanogenesis in B16 melanoma 4A5 cells

Compound	Concentration (μM)						
	0	1	3	10	30	100	IC_{50} (μM)
Inhibition of melanogenesis (%)							
1	0.0 ± 3.6	−3.3 ± 2.7	6.8 ± 2.4	10.6 ± 1.4*	33.0 ± 2.1**	67.8 ± 3.2**	48
2	0.0 ± 2.5	11.6 ± 2.3**	22.5 ± 1.1**	49.3 ± 0.8**	80.1 ± 0.7**	–	10
3	0.0 ± 2.0	7.5 ± 5.8	8.9 ± 3.5	23.4 ± 2.5**	49.2 ± 3.2**	81.1 ± 2.5**	30
4	0.0 ± 0.7	10.4 ± 3.3	11.1 ± 3.0	7.2 ± 13.5	48.1 ± 0.3**	–	ca.33
5	0.0 ± 2.5	0.0 ± 1.5	5.9 ± 1.2	39.9 ± 4.2**	81.2 ± 6.0**	78.4 ± 1.7**	16
6	0.0 ± 3.7	−0.5 ± 4.4	−5.3 ± 2.6	43.4 ± 2.2**	87.1 ± 1.5**	–	17
7	0.0 ± 2.9	–	−5.8 ± 11.3	10.8 ± 7.9	16.9 ± 3.4	47.7 ± 4.4**	>100
8	0.0 ± 4.5	0.1 ± 3.7	6.3 ± 1.8	3.8 ± 2.5	16.9 ± 1.8**	20.9 ± 2.3**	>100
9	0.0 ± 5.1	7.6 ± 7.7	3.7 ± 2.7	6.1 ± 2.6	8.3 ± 1.5	15.5 ± 3.1	>100
10	0.0 ± 4.9	8.3 ± 4.1	9.9 ± 3.4	23.4 ± 2.3**	61.1 ± 3.1**	–	20
11	0.0 ± 2.4	8.7 ± 2.1	1.2 ± 5.2	12.7 ± 2.9*	20.5 ± 3.0**	56.0 ± 2.1**	84
	0	10	30	100	300	1000	IC_{50} (μM)
Inhibition of melanogenesis (%)							
Arbutin	0.0 ± 8.6	6.7 ± 3.4	17.8 ± 4.5	46.9 ± 3.0**	71.0 ± 2.7**	88.7 ± 2.2**	116

Each value represents the mean ± S.E.M. (n = 4).
Significantly different from the control.
* p <0.05
** p <0.01.

Table 3
Effects of constituents (**1**–**9**) isolated from *Alpinia officinarum* and related flavonoids (**10**, **11**) on proliferation of B16 melanoma 4A5 cells

Compound	Concentration (μM)						
	0	1	3	10	30	100	IC_{50} (μM)
Inhibition of proliferation (%)							
1	0.0 ± 0.8	0.0 ± 1.4	0.7 ± 0.5	−4.7 ± 0.9	−7.6 ± 1.0	−7.6 ± 0.9	>100
2	0.0 ± 0.8	3.2 ± 0.7	3.8 ± 0.6	5.1 ± 2.2*	2.6 ± 1.3	90.6 ± 0.1**	ca. 60
3	0.0 ± 2.2	5.3 ± 1.4	11.7 ± 1.4**	11.8 ± 1.2**	15.1 ± 1.5**	20.8 ± 1.5**	>100
4	0.0 ± 1.2	1.1 ± 1.2	−0.5 ± 1.8	1.6 ± 0.8	−2.3 ± 0.6	90.2 ± 1.4**	ca. 60
5	0.0 ± 2.2	−2.0 ± 0.9	3.1 ± 1.1	7.9 ± 0.6**	7.4 ± 0.8**	11.2 ± 1.3**	>100
6	0.0 ± 0.4	1.7 ± 0.5	7.1 ± 2.5	11.4 ± 3.9*	12.7 ± 1.8**	53.3 ± 2.5**ca. 100	
7	0.0 ± 1.9	2.2 ± 0.8	3.6 ± 1.2	7.0 ± 0.9**	7.1 ± 0.4**	6.9 ± 2.0**	>100
8	0.0 ± 1.4	−1.4 ± 1.5	−2.8 ± 1.4	1.2 ± 0.5	1.3 ± 0.1	2.9 ± 1.0	>100
9	0.0 ± 0.4	−0.5 ± 0.6	−2.8 ± 1.1	−8.0 ± 0.5	−8.8 ± 0.5	−6.1 ± 1.7	>100
10	0.0 ± 3.5	5.6 ± 0.9	18.1 ± 3.5**	23.5 ± 4.5**	26.1 ± 1.2**	56.0 ± 0.7**	88
11	0.0 ± 2.5	0.2 ± 1.8	5.9 ± 1.5	13.8 ± 0.6	23.9 ± 5.7**	30.5 ± 0.7**	>100
	0	10	30	100	300	1000	IC_{50} (μM)
Inhibition of proliferation (%)							
Arbutin	0.0 ± 2.1	8.7 ± 2.0	17.6 ± 3.0**	21.9 ± 1.9**	20.2 ± 2.2**	46.9 ± 1.8**	>1000

Each value represents the mean ± S.E.M. (n = 4).
Significantly different from the control.
* p <0.05.
** p <0.01.

diarylheptanoids (**1**–**4**) and two flavonol constituents (**5**, **6**) substantially inhibited melanogenesis with IC_{50} values of 10–48 μM, and an enone moiety in **2** and the 2,3-double bond in **6** are important for more potent activity. In addition, **2**, **5**, and **6** inhibited the mRNA expression of tyrosinase, TRP-1 and -2, and protein levels of the transcriptional factor MITF. The mechanism of action of **2**, **5**, and **6** including target their molecules should be studied further.

4. Materials and methods

4.1. Extraction and isolation of 1–9 from *A. officinarum*

The extraction and isolation of compounds **1**–**9** were described in our previous report.[29] Briefly, the dried rhizomes of *A. officinarum* (3.0 kg) were extracted three times with 80% aqueous acetone at room temperature. The extract (9.6% from this natural medicine) was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble fraction (5.2%) and water-soluble fraction (4.4%). The EtOAc-soluble fraction, which showed potent activity (Table 1), was subjected to normal-phase silica-gel (SiO_2) column chromatography [*n*-hexane→*n*-hexane:EtOAc (9:1→8:2→7:3→1:1→3:7)→EtOAc→MeOH] to give 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhept-4-en-3-one (**2**, 0.43%) and seven fractions (Fr. 1–7). Fractions 3–6 were subjected to reversed-phase silica-gel (ODS) column chromatography (MeOH–H_2O) and finally HPLC (ODS, 250 × 20 mm id, MeOH–H_2O) to give three diarylheptanoids [5-hydroxy-1,7-diphenyl-3-heptanone (**1**, 0.50%), 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**3**, 0.88%), 3,5-dihydroxy-1,7-diphenylheptane (**4**, 0.0005%)], three flavonoids [kaempferide (**5**, 0.017%), galangin (**6**, 1.0%), pinobaksin (**7**, 0.0013%)], 3-phenylpropanoic acid (**8**, 0.004%), and zingerone (**9**, 0.0005%). Kaempferol (**10**) and aromadendrin (**11**) were isolated as described in our previous reports.[31,32]

4.2. Bioassay methods

4.2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM, 4500 mg/L glucose) was purchased from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA); the RNeasy™ Mini Kit was obtained from Qiagen (Huntsville, AL, USA); the Cell Counting Kit-8™ was from Dojindo Lab. (Kumamoto, Japan); illustra™ Ready-To-Go™ RT-PCR Beads, enhanced chemiluminescence kit (ECL plus),

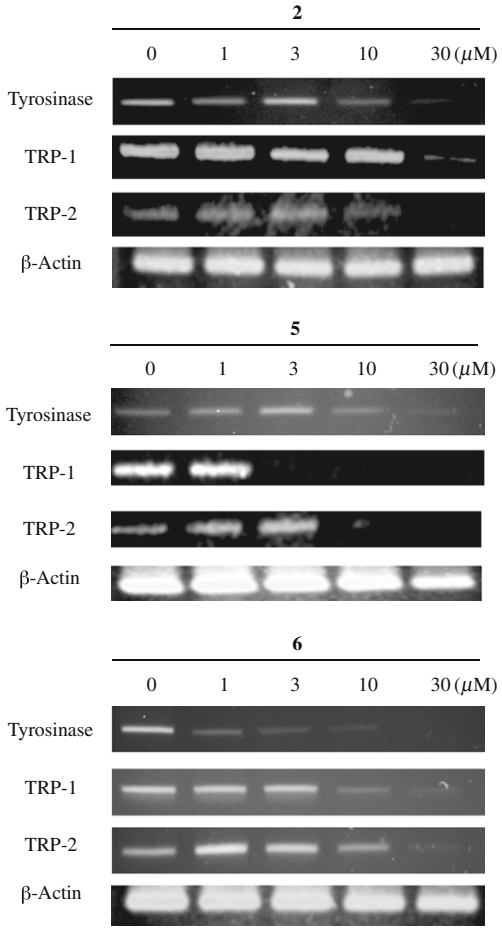

Figure 3. Effects of **2**, **5**, and **6** on mRNA expression of tyrosinase and TRP-1 and -2 in theophylline-stimulated B16 melanoma 4A5 cells. The cells were incubated with or without the test compound for 72 h to detect tyrosinase and TRP-1 and -2 mRNA using RT-PCR. The images are representative of several experiments.

Table 4
Effects of constituents (**1**–**9**) isolated from *Alpinia officinarum* and related flavonoids (**10**, **11**) on mushroom tyrosinase

Compound	Concentration (μM)						
	0	1	3	10	30	100	IC_{50} (μM)
Inhibition of tyrosinase (%)							
1	0.0 ± 3.2	3.3 ± 1.0	2.5 ± 0.6	3.4 ± 1.1	2.4 ± 0.7	2.1 ± 0.2	>100
2	0.0 ± 0.1	3.2 ± 0.3**	3.0 ± 0.4**	3.6 ± 0.4**	5.3 ± 0.3**	7.7 ± 0.5**	>100
3	0.0 ± 0.8	2.3 ± 0.6	4.7 ± 0.7**	5.0 ± 0.6**	6.2 ± 0.3**	10.4 ± 0.3**	>100
4	0.0 ± 1.3	2.7 ± 0.6	4.6 ± 0.7**	4.6 ± 0.6**	5.0 ± 0.8**	0.4 ± 0.4	>100
5	0.0 ± 0.3	−1.7 ± 0.8	−0.2 ± 0.7	0.2 ± 0.6	1.6 ± 0.3	−5.3 ± 1.5	>100
6	0.0 ± 3.7	28.0 ± 2.1**	26.6 ± 3.9**	22.8 ± 2.4**	38.3 ± 3.9**	34.4 ± 3.6**	>100
7	0.0 ± 0.8	1.8 ± 0.5	2.8 ± 0.4*	3.5 ± 1.5**	1.1 ± 0.6	1.1 ± 0.8	>100
8	0.0 ± 0.1	3.8 ± 0.6**	5.3 ± 0.4**	4.9 ± 0.5**	4.8 ± 0.5**	5.6 ± 0.9**	>100
9	0.0 ± 2.0	1.4 ± 1.3	2.1 ± 0.5	0.1 ± 0.6	0.7 ± 1.6	6.1 ± 0.4**	>100
10	0.0 ± 0.8	5.7 ± 0.9	7.6 ± 0.8**	12.1 ± 0.8**	24.2 ± 0.4**	38.4 ± 1.1**	>100
11	0.0 ± 1.0	6.0 ± 1.2**	4.4 ± 0.7	6.3 ± 0.4**	7.9 ± 0.8**	21.0 ± 0.6**	>100
	0	10	30	100	300	1000	IC_{50} (μM)
Inhibition of tyrosinase (%)							
Arbutin	0.0 ± 0.7	0.7 ± 1.6	1.2 ± 1.3	5.6 ± 1.0*	0.1 ± 1.7	10.0 ± 0.9**	>1000
Kojic acid	0.0 ± 1.1	5.4 ± 0.6**	46.2 ± 0.7**	76.0 ± 0.5**	86.8 ± 0.3**	92.5 ± 0.1**	33

Each value represents mean ± S.E.M. ($n = 4$).
Significantly different from the control.
* $p < 0.05$.
** $p < 0.01$.

anti-rabbit IgG, and horseradish peroxidase (HRP)-linked whole antibody from donkeys were from GE Healthcare (Buckinghamshire, UK); Blocking One was from Nacalai Tesque (Kyoto, Japan); the phosphatase inhibitor was from Roche (Mannheim, Germany); the proteinase inhibitor and Protein Assay Kit were from Thermo Scientific (MA, USA); rabbit polyclonal antibody against anti-microphthalmia-associated transcription factor (anti-MITF) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and the other chemicals were purchased from Wako Pure Chemical Co., Ltd (Osaka, Japan). The 6- and 24-multiplates and 96-well microplates (Sumilon) were purchased from Sumitomo Bakelite Co., Ltd (Tokyo, Japan).

4.2.2. Cell culture

Murine B16 melanoma 4A5 cells (RCB0557) were obtained from Riken Cell Bank (Tsukuba, Japan), and the cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2/air. The cells were harvested by incubation in phosphate-buffered saline (PBS) containing EDTA 1 mM and 0.25% trypsin for ca. 5 min at 37 °C and were used for the subsequent bioassays.

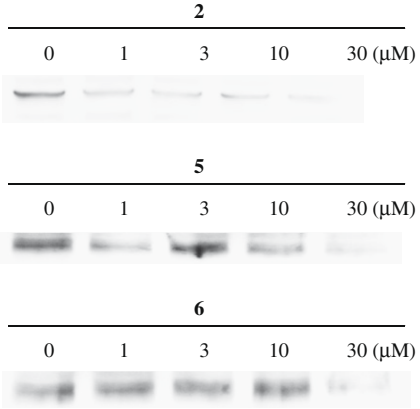

Figure 4. Effects of **2**, **5**, and **6** on protein levels of microphthalmia-associated transcription factor (MITF) in theophylline-stimulated B16 melanoma 4A5 cells. The cells were incubated with or without the test compound for 48 h, and then MITF protein levels were detected using SDS–PAGE and Western blotting. The images are representative of several experiments.

4.2.3. Cell viability

The melanoma cells (4.0×10^3 cells/100 μL/well) were seeded into 96-well microplates and incubated for 24 h. After 68-h incubation with theophylline 1 mM and a test compound, 10 μL of WST-8 solution (Cell Counting Kit-8™) was added to each well. After a further 4 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) at 450 nm (reference: 655 nm). The test compound was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the medium was 0.1%. Cell viability (%) and inhibition (%) were calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{Cell viability } (\%) = A/B \times 100$$
$$\text{Inhibition } (\%) = (A - B)/A \times 100$$

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively.

4.2.4. Melanogenesis

The melanoma cells (1.6×10^4 cells/ 400 μL/well) were seeded into 24-well multiplates. After 24 h of culture, a test compound and theophylline 1 mM were added and incubated for 72 h. The cells were harvested by incubating with PBS containing EDTA 1 mM and 0.25% trypsin, and then the cells were washed with PBS. The cells were treated with NaOH 1 M (120 μL/tube, 80 °C, 30 min) to yield a lysate, an aliquot (100 μL) of the lysate was transferred to a 96-well microplate, and the optical density of each well was measured with a microplate reader (Model 550, Bio-Rad) at 405 nm. The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. The production of melanin was corrected based on cell viability. Inhibition (%) was calculated using the following formula, and IC_{50} values were determined graphically.

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

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively, and C indicates cell viability (%).

4.2.5. Mushroom tyrosinase

The incubation mixture contained 1.8 mL of phosphate buffer (0.1 M, pH 6.5), 1 mL of L-DOPA (2.5 mM in the phosphate buffer) as a substrate, and 100 μL of the test compound solution. The reaction was initiated by the addition of 100 μL of tyrosinase (46 units) at 25 °C for 5 min, and then the absorbance at 405 nm was measured (model DU530, Beckman). The amount of dopaquinone was determined based on the absorbance. The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. The following equation was used to calculate the inhibition of mushroom tyrosinase

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

where A and B indicate the absorbance of vehicle- and test compound-treated groups, respectively.

4.2.6. RT-PCR

The melanoma cells (8×10^4 cells/2 mL/well) were seeded into 6-well multiplates and cultured for 24 h. The cells were then incubated with the test compound and theophylline 1 mM for 72 h. Total RNA was extracted from the cells incubated with the test compound using an RNeasy™ mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and determining the ratio of the readings at 260 nm and 280 nm. cDNA synthesis and the PCR reaction were performed using illustra™ Ready-To-Go™ RT-PCR Beads (GE Healthcare) according to the manufacturer's instructions. The PCR conditions were 28 cycles for tyrosinase, 20 cycles for both TRP-1 and -2, and 25 cycles for β-actin, consisting of incubation at 94 °C for 30 s and then at 55 °C for 1 min and at 72 °C for 2 min. PCR products were electrophoresed in 2% agarose gels and visualized by ethidium bromide staining. The primer pairs were: tyrosinase primers, 5′-CAGATCTCTGATGGCC AT-3′ and 5′-GGATGACATAGACTGAGC-3′; TRP-1, 5′-CTTTCTCCCTT CCTTACTGG; 5′-TGGCTTCATTCTTGGTGCTT-3′; TRP-2, 5′-TGAGAAG AAACAAAGTAGGCAGAA-3′ and 5′-CAACCCCAAGAGCAAGACGAAA GC-3′; and β-actin primers, 5′-GGGAAATCGTGCGTGACAT-3′ and 5′-CAGGAGGAGCAATGATCTC-3′.

4.2.7. Immunoblotting

The melanoma cells (8×10^4 cells/2 mL/well) were seeded into 6-well multiplates and cultured for 24 h. The cells were then incubated with theophylline 1 mM and the test compound for 24 h or 48 h. After incubation, the cells were lysed in lysis buffer containing a phosphatase inhibitor (Roche) and a proteinase inhibitor (Thermo Scientific). Protein concentrations of cell lysates were determined using the Protein Assay Kit (Thermo Scientific). Equivalent amounts of protein (50 μg of protein/lane) were electrophoresed in 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 30 min in Blocking One (Nacalai Tesque). The blots were probed with primary antibody at the appropriate dilution in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 1 h. The membranes were then washed three times with T-TBS and incubated with the appropriate HRP-conjugated secondary antibody for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL plus, GE Healthcare), according to the manufacturer's instructions.

The following antibodies and dilutions were used for Western blotting: rabbit polyclonal antibody against anti-MITF (1:1000) (Santa Cruz Biotechnology); anti-rabbit IgG; and HRP-linked whole antibody from donkeys (1:5000) (GE Healthcare).

4.2.8. Statistical analyses

Values are expressed as mean ± S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analyses.

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References and notes

1. Hearing, V. J.; Körner, A. M.; Pawelek, J. M. *J. Invest. Dermatol.* **1982**, *79*, 16–18.
2. Hearing, V. J.; Jiménez, M. *Int. J. Biochem.* **1987**, *19*, 1141–1147.
3. Prota, G. *Med. Res. Rev.* **1988**, *8*, 525–556.
4. Kuzumaki, T.; Matsuda, A.; Wakamatsu, K.; Ito, S.; Ishikawa, K. *Exp. Cell. Res.* **1993**, *207*, 33–40.
5. Mishima, Y. *Pigment Cell Res.* **1994**, *7*, 376–387.
6. Virador, V.; Matsunaga, N.; Matsunaga, J.; Valencia, J.; Oldham, R. J.; Kameyama, K.; Peck, G. L.; Ferrans, V. J.; Vieira, W. D.; Abdel-Malek, Z. A.; Hearing, V. J. *Pigment Cell Res.* **2001**, *14*, 289–297.
7. Pérez-Bernal, A.; Muñoz-Pérez, M. A.; Camacho, F. *Am. J. Clin. Dermatol.* **2000**, *1*, 261–268.
8. Parvez, S.; Kang, M.; Chung, H. S.; Cho, C.; Hong, M. C.; Shin, M. K.; Bae, H. *Phytother. Res.* **2006**, *20*, 921–934.
9. Ly, T. N.; Yamauchi, R.; Kato, K. *Food Sci. Technol. Res.* **2001**, *7*, 303–306.
10. Inoue, T.; Shinbori, T.; Fujioka, M.; Hashimoto, K.; Masada, Y. *Yakugaku Zasshi* **1978**, *98*, 1255–1257.
11. Itokawa, H.; Morita, M.; Mihashi, S. *Chem. Pharm. Bull.* **1981**, *29*, 2383–2385.
12. Hashimoto, T.; Tori, M.; Asakawa, Y. *Chem. Pharm. Bull.* **1986**, *34*, 1846–1849.
13. Itokawa, H.; Morita, H.; Midorikawa, I.; Aiyama, R.; Morita, M. *Chem. Pharm. Bull.* **1985**, *33*, 4889–4893.
14. Kiuchi, F.; Shibuya, M.; Sankawa, U. *Chem. Pharm. Bull.* **1982**, *30*, 2279–2282.
15. Uehara, S.; Yasuda, I.; Akiyama, K.; Morita, H.; Takeya, K.; Itokawa, H. *Chem. Pharm. Bull.* **1987**, *35*, 3298–3304.
16. Shin, D.; Kinoshita, K.; Koyama, K.; Takahashi, K. *J. Nat. Prod.* **2002**, *65*, 1315–1318.
17. Kuroyanagi, M.; Yamamoto, Y.; Fukushima, S.; Ueno, A.; Noro, T.; Miyase, T. *Chem. Pharm. Bull.* **1982**, *30*, 1602–1608.
18. Bleier, W.; Chirikdjian, J. J. *Planta Med.* **1972**, *22*, 145–151.
19. Shin, J. E.; Han, M. J.; Kim, D. H. *Biol. Pharm. Bull.* **2003**, *26*, 854–857.
20. Ly, T. N.; Yamauchi, R.; Shimoyamada, M.; Kato, K. *J. Argric. Food Chem.* **2002**, *50*, 4919–4924.
21. Ly, T. N.; Shimoyamada, M.; Kato, K.; Yamauchi, R. *Biofactors* **2004**, *21*, 305–308.
22. Ly, T. N.; Shimoyamada, M.; Kato, K.; Yamauchi, R. *J. Agric. Food Chem.* **2003**, *51*, 4924–4929.
23. Heo, M. Y.; Sohn, S. J.; Au, W. W. *Mutat. Res.* **2001**, *488*, 135–150.
24. Kiuchi, F.; Iwakami, S.; Shibuya, M.; Hanaoka, F.; Sankawa, U. *Chem. Pharm. Bull.* **1992**, *40*, 387–391.
25. Shin, J. E.; Han, M. J.; Song, M. C.; Baek, N. I.; Kim, D. H. *Biol. Pharm. Bull.* **2004**, *27*, 138–140.
26. Kim, Y. U.; Son, H. K.; Song, H. K.; Ahn, M. J.; Lee, S. S.; Lee, S. K. *Planta Med.* **2003**, *69*, 72–74.
27. Yadav, P. N.; Liu, Z.; Rafi, M. M. *J. Pharmacol. Exp. Ther.* **2003**, *305*, 925–931.
28. Asakawa, Y. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 575.
29. Matsuda, H.; Ando, S.; Kato, T.; Morikawa, T.; Yoshikawa, M. *Bioorg. Med. Chem.* **2006**, *14*, 138–142.
30. Lu, Y. H.; Lin-Tao; Wang, Z. T.; Wei, D. Z.; Xian, H. B. *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 433–438.
31. Matsuda, H.; Morikawa, T.; Toguchida, I.; Yoshikawa, M. *Chem. Pharm. Bull.* **2002**, *50*, 788–795.
32. Yoshikawa, M.; Xu, F.; Morikawa, T.; Ninomiya, K.; Matsuda, H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1045–1049.
33. Friedmann, P. S.; Gilchrest, B. A. *J. Cell. Physiol.* **1987**, *133*, 88–94.
34. Hunt, G.; Todd, C.; Cresswell, J. E.; Thody, A. J. *J. Cell. Sci.* **1994**, *107*, 205–211.
35. Wong, G.; Pawelek, J. *Nature* **1975**, *255*, 644–646.
36. Steinberg, M. L.; Whittaker, J. R. *J. Cell. Physiol.* **1976**, *87*, 265–276.
37. Cho, S. M.; Kwon, Y. M.; Lee, J. H.; Yon, K. H.; Lee, M. W. *Arch. Pharm. Res.* **2002**, *25*, 885–888.
38. Akazawa, H.; Akihisa, T.; Taguchi, Y.; Banno, N.; Yoneima, R.; Yasukawa, K. *Biol. Pharm. Bull.* **2006**, *29*, 1970–1972.
39. Choi, M. Y.; Song, H. S.; Hur, H. S.; Sim, S. S. *Arch. Pharm. Res.* **2008**, *31*, 1166–1171.
40. Nitoda, T.; Isobe, T.; Kubo, I. *Phytother. Res.* **2008**, *22*, 867–872.
41. Kubo, I.; Nitoda, T.; Nihei, K. *Molecules* **2007**, *12*, 1045–1056.
42. Yoon, H. S.; Lee, S. R.; Ko, H. C.; Choi, S. Y.; Park, J. G.; Kim, J. K.; Kim, S. J. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 1781–1784.
43. Kubo, I.; Kinst-Hori, I. *J. Agric. Food Chem.* **1999**, *47*, 4121–4125.
44. Bertolotto, C.; Busca, R.; Abbe, P.; Bille, K.; Aberdam, E.; Ortonne, J. P.; Ballotti, R. *Mol. Cell. Biol.* **1998**, *18*, 694–702.
45. Tachibana, M. *J. Invest. Dermatol. Symp. Proc.* **2001**, *6*, 95–98.
46. Levy, C.; Khaled, M.; Fisher, D. E. *Trends Mol. Med.* **2006**, *12*, 406–414.
47. Kim, Y. J.; No, J. K.; Lee, J. S.; Kim, M. S.; Chung, H. Y. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 532–534.
48. Maeda, K.; Naitou, T.; Umishio, K.; Fukuhara, T.; Motoyama, A. *Biol. Pharm. Bull.* **2007**, *30*, 873–879.