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Melanogenesis inhibitors from the desert plant *Anastatica hierochuntica* in B16 melanoma cells

Souichi Nakashima, Hisashi Matsuda, Yoshimi Oda, Seikou Nakamura, Fengming Xu, Masayuki Yoshikawa *

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan

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ABSTRACT

The methanolic extract from the whole plants of *Anastatica hierochuntica*, an Egyptian herbal medicine, was found to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells. Among the constituents isolated, anastatin A, silybin A, isosilybins A and B, eriodictyol, luteolin, kaempferol, quercetin, hierochins A and B, (2*R*,3*S*)-2,3-dihydro-2-(3,4-dimethoxyphenyl)-3-hydroxymethyl-5-(2-formylvinyl)-7-hydroxybenzofuran, (+)-dehydrodiconiferyl alcohol, (+)-balanophonin, 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(*E*)-3-hydroxy-1-propenyl]-2-methoxyphenoxy}-1,3-propanediol, and 3,4-dihydroxybenzaldehyde substantially inhibited melanogenesis with IC_{50} values of 6.1–32 μM. With regard to the mechanism of action of silybins and isosilybins, the inhibition of tyrosinase activity suggested to be important. In addition, isosilybins A and B inhibited the mRNA expression of TRP-2, but silybins A and B oppositely enhanced the mRNA expression of tyrosinase and TRP-1 and -2 at 10 and/or 30 μM, and the inhibition of phosphorylation of extracellular signal-regulated kinases (ERK1/2) is involved in the enhanced expression of mRNA, at least in part, similar to that of PD98059.

1. Introduction

The Cruciferae plant, *Anastatica hierochuntica*, is widely distributed in the Sahara-Arabian desert areas and the whole plants of *A. hierochuntica* are prescribed for the treatment of fatigue and uterine hemorrhage in Egyptian folk medicine and are used by women as a charm for child birth.[1,2] During the course of our studies on bioactive constituents of Egyptian herbal medicines,[3–13] we reported the structural elucidation of the new skeletal benzofuranoflavanones, anastains A (**1**) and B (**2**), with hepatoprotective activities and new (7*R*,8*S*) and (7*S*,8*R*) 8–5′ linked neolignans, hierochins A (**19**), B (**24**), and C (**21**), from this herbal medicine.[7,8]

Melanin biosynthesis proceeds through a complex series of enzymatic and chemical reactions in melanocytes.[14–17] 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.[15] This tyrosinase process is involved in abnormal accumulation of melanin pigments (hyperpigmentation).[18,19] Therefore, tyrosinase inhibitors such as kojic acid and arbutin have been established as important constituents of cosmetic products and depigmenting agents for the treatment of hyperpigmentation.[20,21]

We have been searching for inhibitors of melanin production from natural medicines to develop the constituents of cosmetic products and depigmenting agents.[22] As a continuing study, the methanolic (MeOH) extract from the whole plants of *A. hierochuntica*, an Egyptian herbal medicine, was found to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells.

In this paper, we describe the effects of constituents (**1**–**44**) from the whole plant of *A. hierochuntica* on melanogenesis in the cells and the effects of silybins A (**5**) and B (**6**) and isosilybins A (**7**) and B (**8**), which showed the inhibitory effects on melanogenesis, on mRNA expressions of tyrosinase and TRP-1 and -2.

2. Results and discussion

2.1. Effects on melanogenesis in B16 melanoma 4A5 cells

Melanocytes can be stimulated by many effectors including ultraviolet radiation[23] and α-melanocyte-stimulating hormone (α-MSH).[24] 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.[24,25] In the present study, we used the phosphodiesterase inhibitor theophylline[26] to stimulate B16 melanoma 4A5 cells. As shown in Table 1, the methanolic extract significantly inhibited melanogenesis with an IC_{50} value of 100 μg/mL.

The MeOH ext. (153 g) was suspended in water (H_2O) and then extracted with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) successively to give EtOAc- (70 g, 2.4%), *n*-BuOH- (18 g, 0.6%) and

* Corresponding author. Tel.: +81 75 595 4633; fax: +81 75 595 4768.
E-mail address: myoshika@mb.kyoto-phu.ac.jp (M. Yoshikawa).

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Table 1
Inhibitory effects of MeOH ext. and EtOAc-, *n*-BuOH- and H_2O-soluble fractions and the isolated compounds from *A. hierochuntica* on melanogenesis and proliferation in B16 melanoma 4A5 cells

	Concentration (μg/mL)						IC_{50} (μg/mL)
	0	1	3	10	30	100	
Inhibition of melanogenesis (%)							
MeOH ext.	0.0 ± 3.2	4.1 ± 1.2	4.2 ± 1.6	4.1 ± 2.1	14.7 ± 1.8**	49.6 ± 0.7**	100
EtOAc-soluble fr.	0.0 ± 3.2	1.5 ± 3.9	−2.2 ± 2.4	9.8 ± 2.0	26.8 ± 1.6**	66.7 ± 5.3**	60
n-BuOH-soluble fr.	0.0 ± 1.8	4.6 ± 4.4	0.3 ± 4.5	−9.7 ± 3.1	−8.8 ± 3.4	−33.3 ± 4.3**	–
H_2O-soluble fr.	0.0 ± 3.6	1.4 ± 1.1	−0.8 ± 4.1	−1.0 ± 4.1	−6.4 ± 2.6	0.5 ± 1.6	–

	Concentration (μM)					IC_{50} (μM)
	0	1	3	10	30	
Inhibition of melanogenesis (%)[a]						
Anastatin A (**1**)	0.0 ± 2.3	7.1 ± 1.0	14.9 ± 2.5**	35.9 ± 2.3**	65.1 ± 1.5**	16
Anastatin B (**2**)	0.0 ± 4.7	−1.1 ± 2.7	10.1 ± 2.8	19.5 ± 1.9**	40.3 ± 2.6**	–
(+)-Silychristin (**3**)	0.0 ± 2.5	4.1 ± 2.1	1.3 ± 5.4	3.8 ± 2.1	17.4 ± 2.6**	–
(−)-Silychristin (**4**)	0.0 ± 1.3	4.2 ± 2.0	−1.3 ± 2.6	1.6 ± 2.3	1.2 ± 2.0	–
Silybin A (**5**)	0.0 ± 3.9	9.2 ± 4.3	18.8 ± 1.5**	24.4 ± 3.8**	64.7 ± 4.0**	21
Silybin B (**6**)	0.0 ± 2.3	−0.2 ± 4.4	3.0 ± 4.1	10.2 ± 1.2	33.0 ± 4.8**	–
Isosilybin A (**7**)	0.0 ± 2.4	11.7 ± 5.2	19.8 ± 3.5**	50.2 ± 3.3**	72.1 ± 2.8**	10
Isosilybin B (**8**)	0.0 ± 6.3	18.7 ± 3.6*	37.5 ± 4.9**	58.0 ± 3.3**	73.4 ± 3.6**	6.1
Naringenin (**9**)	0.0 ± 3.0	14.4 ± 1.6**	13.9 ± 2.0**	22.5 ± 3.0**	16.9 ± 2.4**	–
Eriodictyol (**10**)[b]	0.0 ± 4.2	6.5 ± 2.9	10.5 ± 2.4	32.3 ± 2.1**	46.3 ± 1.8**	32
(+)-Aromadendrin (**11**)	0.0 ± 2.4	6.0 ± 2.5	14.4 ± 2.7**	−5.2 ± 1.1	7.0 ± 1.1	–
(+)-Taxifolin (**12**)	0.0 ± 3.8	3.5 ± 2.9	3.1 ± 2.4	6.7 ± 2.8	21.7 ± 2.7**	–
(+)-3′-*O*-Methyltaxifolin (**13**)	0.0 ± 3.0	9.8 ± 5.6	8.0 ± 3.5	10.6 ± 2.1	16.2 ± 4.2*	–
(+)-Epitaxifolin (**14**)	0.0 ± 1.1	3.5 ± 1.9	6.8 ± 3.7	6.9 ± 1.9	13.7 ± 2.5**	–
Luteolin (**15**)	0.0 ± 4.0	6.2 ± 4.4	13.5 ± 1.4*	38.1 ± 1.2**	76.7 ± 0.9**	14
Kaempferol (**16**)	0.0 ± 3.0	−0.6 ± 4.1	7.9 ± 4.5	18.6 ± 3.3**	58.1 ± 1.8**	25
Quercetin (**17**)	0.0 ± 1.2	13.9 ± 1.4**	17.1 ± 1.3**	34.3 ± 2.2**	72.5 ± 1.4**	15
Rutin (**18**)	0.0 ± 1.7	4.5 ± 0.7*	3.1 ± 0.6	4.3 ± 1.0*	5.2 ± 1.2*	–
Hierochin A (**19**)	0.0 ± 3.2	1.9 ± 3.5	8.0 ± 2.0	32.5 ± 2.1**	51.8 ± 1.5**	25
20	0.0 ± 6.2	10.7 ± 5.1	8.4 ± 4.1	33.6 ± 4.3**	76.8 ± 4.0**	21
Hierochin C (**21**)	0.0 ± 1.9	−1.0 ± 2.8	6.6 ± 4.4	27.6 ± 2.0**	44.6 ± 4.1**	–
(+)-Dehydrodiconiferyl alcohol (**22**)	0.0 ± 0.7	−1.6 ± 2.7	−5.4 ± 2.6	18.6 ± 3.2**	72.7 ± 0.7**	16
(+)-Balanophonin (**23**)	0.0 ± 1.9	−2.8 ± 4.9	2.1 ± 0.6	28.4 ± 3.5**	79.1 ± 2.6**	15
Hierochin B (**24**)	0.0 ± 4.5	−1.6 ± 2.1	2.2 ± 1.7	7.6 ± 3.3	50.6 ± 1.9**	30
26	0.0 ± 3.5	14.9 ± 2.3**	20.8 ± 4.0**	21.3 ± 1.3**	27.3 ± 3.6**	–
(−)-Evofolin B (**28**)	0.0 ± 1.7	18.2 ± 3.8**	15.0 ± 1.3*	16.4 ± 3.2**	23.5 ± 4.5**	–
29	0.0 ± 2.4	8.8 ± 1.9*	11.7 ± 2.0**	24.5 ± 2.3**	52.9 ± 1.9**	29
3,4-Dihydroxybenzaldehyde (**36**)	0.0 ± 5.9	8.5 ± 4.5	13.8 ± 2.3	40.4 ± 2.7**	57.9 ± 1.9**	17
Acetovanillone (**38**)	0.0 ± 2.6	16.8 ± 4.2**	17.5 ± 2.6**	18.9 ± 2.4**	20.2 ± 3.1**	–
41	0.0 ± 2.9	16.5 ± 2.1**	18.1 ± 2.7**	24.0 ± 3.5**	25.8 ± 3.7**	–
Coniferaldehyde (**44**)	0.0 ± 1.0	10.3 ± 2.1*	11.6 ± 2.8*	14.0 ± 3.4**	39.1 ± 2.0**	–

	Concentration (μM)						IC_{50} (μM)
	0	10	30	100	300	1000	
Inhibition of melanogenesis (%)							
Arbutin	0.0 ± 1.4	10.6 ± 0.6**	20.4 ± 0.5**	38.1 ± 0.9**	61.5 ± 0.6**	83.7 ± 0.5**	174

The melanoma cells (2.0×10^4 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 and 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, and the optical density of the lysate was measured at 405 nm (reference: 655 nm). The production of melanin was corrected based on cell viability.

Each value represents the mean ± S.E.M. ($n = 4$).

Significantly different from the control group, *p <0.05, **p <0.01.

[a] Inhibitions by **25**, **27**, **31**–**35**, **37**, **39**, **40**, **42**, and **43** at 30 μM and **30** at 10 μM were less than 15.4%.

[b] Inhibition by **10** at 100 μM was 70.5 ± 2.3% (p <0.01).

H_2O-soluble fractions (63 g, 2.2%), respectively. The EtOAc-soluble fraction, which showed a significant inhibition (IC_{50} value = 60 μg/mL) without cytotoxic effect less than 100 μg/mL (data not shown), and *n*-BuOH-soluble fraction were respectively subjected to ordinary- and reversed-phase silica gel column chromatographies and finally HPLC to give the following known compounds: anastatins A (**1**,[7] 0.0010% from this natural medicine) and B (**2**,[7] 0.00098%), (+)-silychristin (**3**,[27] 0.0011%), (−)-silychristin (**4**,[27] 0.00073%), silybins A (**5**,[28] 0.0011%) and B (**6**,[28] 0.0014%), isosilybins A (**7**,[28] 0.0015%) and B (**8**,[28] 0.00090%), naringenin (**9**,[29] 0.0038%), eriodictyol (**10**,[30] 0.0027%), (+)-aromadendrin (**11**,[31] 0.00081%), (+)-taxifolin (**12**,[32] 0.044%), 3′-*O*-methyltaxifolin (**13**,[33] 0.00038%), (+)-epitaxifolin (**14**,[32] 0.0035%), luteolin (**15**,[34] 0.00079%), kaempferol (**16**,[34] 0.00060%), quercetin (**17**,[34] 0.0010%), rutin (**18**,[34] 0.00072%), hierochins A (**19**,[8] 0.00046%), B (**24**,[8] 0.00029%), and C (**21**,[8] 0.00070%), hierochin D[35] (0.0010%), (2*R*,3*S*)-2,3-dihydro-2-(3,4-dimethoxyphenyl)-3-hydroxymethyl-5-(2-formylvinyl)-7-hydroxybenzofuran (**20**,[36] 0.0061%), (+)-dehydrodiconiferyl alcohol (**22**,[37] 0.0011%), (+)-balanophonin (**23**,[38] 0.00045%), (+)-lariciresinol (**25**,[39] 0.0024%), (+)-1,2-bis-(4-hydroxy-3-methoxyphenyl)-propane-1,3-diols [*erythro* form (**26**,[40,41] 0.0029%) and *threo* form (**27**,[40,41] 0.0011%)], (−)-evofolin B (**28**,[42] 0.00093%), 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(*E*)-3-hydroxy-1-propenyl]-2-methoxyphenoxy}-1,3-propanediol (**29**,[43] 0.00056%), 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[2-formyl-(*E*)-vinyl]-2-methoxyphenoxy}-1,3-propanediol (**30**,[40] 0.00019%), *p*-hydroxybenzoic

acid (**31**,[44] 0.0012%), *p*-methoxybenzoic acid (**32**,[44] 0.00075%), 3,4-dihydroxybenzoic acid (**33**,[44] 0.0025%), 3-methoxy-4-hydroxybenzoic acid (**34**,[44] 0.0068%), *p*-hydroxybenzaldehyde (**35**,[34] 0.00093%), 3,4-dihydroxybenzaldehyde (**36**,[45] 0.0016%), vanillin (**37**,[46] 0.0036%), acetovanillone (**38**,[46] 0.00066%), 2,4′-dihydroxy-3′-methoxyacetophenone (**39**,[47] 0.0011%), ω-hydroxypropioguaiacone (**40**,[48] 0.0015%), (+)-2,3-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (**41**,[49] 0.0015%), *trans*-cinnamic acid (**42**,[50] 0.00059%), *trans*-ferulic acid (**43**,[51,52] 0.00079%), coniferaldehyde (**44**,[53] 0.0013%), and β-sitosterol 3-*O*-β-D-glucopyranoside[54] (0.0025%) (Fig. 1).

Among the constituents isolated, anastatin A (**1**), silybin A (**5**), isosilybins A (**7**) and B (**8**), eriodictyol (**10**), luteolin (**15**), kaempferol (**16**), quercetin (**17**), hierochins A (**19**) and B (**24**), **20**, (+)-dehydrodiconiferyl alcohol (**22**), (+)-balanophonin (**23**), **29**, and 3,4-dihydroxybenzaldehyde (**36**) substantially inhibited the melanogenesis with IC_{50} values of 6.1–32 μM. These active compounds showed greater activity than arbutin (IC_{50} = 174 μM) (Table 1). In addition, moderate cytotoxic effects were observed after incubation with **5**, **6**, **15**, and **22–24** at a high concentration (more than 20% inhibition at 30 μM), and **30** showed a greater cytotoxic effect (IC_{50} = 6.9 μM) (Table 2).

We previously reported that flavonols such as kaempferol (**16**) inhibited the melanogenesis, but the corresponding dihydroflavonol, (+)-aromadendrin (**11**) showed less inhibition.[22] In agreement with the previous report,[22] a flavone (**15**) and two flavonols (**16**, **17**) inhibited melanogenesis in the cells. However, two flavanones (**9**, **10**), three flavanonols (**11**–**14**), and a flavonol having the 3-*O*-glycoside (**18**) showed less inhibition. These findings suggest that 2,3-double bond is essential for the greater activity,[22] and the 3-*O*-glycoside moiety reduced the activity of flavonols. Furthermore, the 3′,4′-dihydroxyl group is important for the activity [**10** (46.3% inhibition at 30 μM) > **9** (16.9% inhibition at 30 μM); **12** (21.7% inhibition at 30 μM) > **11** (7.0% inhibition at 30 μM); **17** (IC_{50} = 15 μM) > **16** (25 μM)].

Luteolin (**15**) was already reported to inhibit tyrosinase activity and melanin production by inhibiting adenyl cyclase in α-MSH-stimulated B16 melanoma cells.[55] In addition, Fuji and Saito reported that quercetin (**17**) also inhibited melanogenesis in B16 melanoma cells by inhibiting tyrosinase and tyrosinase protein levels.[56] On the other hand, several flavonoids such as **17**, pedalitin, and nobiletin were reported not to suppress the production of melanin but rather to enhance it.[57–59] In the present study, **17** significantly inhibited the melanogenesis (IC_{50} = 15 μM) under our experimental conditions.

Anastatin A (**1**, IC_{50} = 16 μM) exhibited greater activity than naringenin (**9**, >100 μM) and anastatin B (**2**, >100 μM), suggesting that benzofuran moiety enhanced the activity of flavanones, but the position of it is important for the activity. Silybin A (**5**, IC_{50} = 21 μM) and isosilybins A (**7**, 10 μM) and B (**8**, 6.1 μM) showed greater inhibitions than (+)-taxifolin (**12**, >100 μM) and silybin B (**6**, >100 μM). Especially, **8** greatly inhibited the melanogenesis without cytotoxic effects. These results indicate that the 3-methoxy-4-hydroxyphenylpropanol moiety enhances the activity of flavanonols but the position and conformation are also important for the activity [**8** (IC_{50} = 6.1 μM) > **7** (10 μM) > **5** (21 μM) > **6** (33.0% inhibition at 30 μM) > **3** (17.4% inhibition at 30 μM) > **4** (1.2% inhibition at 30 μM)].

2.2. Effects on mushroom tyrosinase

Mushroom tyrosinase has conventionally been used for the development of tyrosinase inhibitors.[20] In the present study, a positive control, kojic acid, substantially inhibited the enzyme activity with IC_{50} value of 30 μM, and silybin A (**5**), isosilybins A (**7**) and B (**8**), kaempferol (**16**), and quercetin (**17**) showed moderate inhibitory activity under our experimental conditions [inhibition (%): 24.2%, 29.4%, 30.8%, 24.2%, and 45.8%, respectively, at a concentration of 30 μM], but other inhibitors of melanogenesis showed weak inhibition (less than 20% inhibition at 30 μM) (Table 3).

2.3. Effects on melanin production from dopachrome by autoxidation

Next, we examined effects of the active constituents on melanin production from dopachrome by autoxidation.[60,61] The positive control, kojic acid, inhibited the autoxidation with IC_{50} value of 250 μM. However, the compounds **1**, **5**, **7**, **8**, **10**, **15**–**17**, **19**, **20**, **22**, **23**, **29**, and **36** which exhibited the melanogenesis in the cells did not show such effect at 100 μM (data not shown).

2.4. Effects of silybins A (5) and B (6) and isosilybins A (7) and B (8) on mRNA expression of tyrosinase and TRP-1 and -2, and on phosphorylation of ERK1/2

The tyrosinase-related protein 1 (TRP-1) and TRP-2 as well as tyrosinase are known to be enzymes catalyzing the major steps

Table 2
Inhibitory effects of compounds **1**–**44** from *A. hierochuntica* on proliferation in B16 melanoma 4A5 cells

	Concentration (μM)					IC_{50} (μM)
	0	1	3	10	30	
Inhibition of proliferation (%)[a]						
Silybin A (**5**)	0.0 ± 2.9	−5.7 ± 0.8	−10.7 ± 2.0**	7.5 ± 1.3*	33.8 ± 0.6**	>100
Silybin B (**6**)	0.0 ± 2.4	−2.0 ± 1.2	−1.4 ± 1.1	11.8 ± 1.4**	44.4 ± 2.1**	>100
Isosilybin A (**7**)	0.0 ± 2.2	−2.2 ± 2.0	−1.5 ± 1.3	−2.5 ± 0.6	16.0 ± 1.7**	—
Luteolin (**15**)	0.0 ± 1.5	−4.8 ± 0.9*	−4.9 ± 1.0*	4.0 ± 1.6	23.4 ± 0.4**	>100
Kaempferol (**16**)	0.0 ± 2.3	3.0 ± 2.0	10.2 ± 0.3**	13.0 ± 1.1**	16.3 ± 1.9**	—
Quercetin (**17**)	0.0 ± 2.3	−8.8 ± 1.3**	−7.7 ± 1.4**	−8.6 ± 0.7**	11.9 ± 0.7**	—
(+)-Dehydrodiconiferyl alcohol (**22**)	0.0 ± 1.9	−2.7 ± 2.3	0.3 ± 1.1	−1.6 ± 2.7	23.3 ± 4.8**	>100
(+)-Balanophonin (**23**)	0.0 ± 0.8	3.8 ± 1.9	5.5 ± 2.2	13.6 ± 2.2**	40.3 ± 1.2**	>100
Hierochin B (**24**)	0.0 ± 1.2	0.3 ± 0.8	1.3 ± 0.6	10.9 ± 0.8**	23.8 ± 1.2**	>100
30	0.0 ± 2.9	2.2 ± 2.6	13.6 ± 0.8**	68.6 ± 1.3**	91.5 ± 0.2**	6.9
p-Hydroxybenzaldehyde (**35**)	0.0 ± 2.4	−8.4 ± 2.2*	−13.6 ± 2.8**	−21.0 ± 1.9**	17.6 ± 1.1**	—
3,4-Dihydroxybenzaldehyde (**36**)	0.0 ± 1.0	−4.7 ± 1.0	−4.7 ± 2.5	−3.7 ± 2.6	11.4 ± 1.1**	—
Coniferaldehyde (**44**)	0.0 ± 1.4	−4.2 ± 1.1	−3.2 ± 1.1	−0.5 ± 0.8	11.5 ± 1.3**	—

The melanoma cells (5.0×10^3 cells/100 μL/well) were seeded into 96-well microplates and incubated for 24 h. After 70-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 2 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader at 450 nm (reference: 655 nm).
Each value represents the mean ± S.E.M. (n = 4).
Significantly different from the control group, *p <0.05, **p <0.01.
[a] The inhibition by **1**–**4**, **8**–**14**, **18**–**21**, **25**–**29**, **31**–**34**, and **37**–**43** at 30 μM were less than 8.8%.

Table 3
Inhibitory effects of selected compounds from *A. hierochuntica* on the enzyme activity of mushroom tyrosinase

	Concentration (μM)						IC_{50} (μM)
	0	1	3	10	30	100	
Inhibition of tyrosinase (%)[a]							
Anastatin A (**1**)	0.0 ± 1.0	6.4 ± 1.3**	6.7 ± 0.5**	9.6 ± 1.0**	11.2 ± 0.5**	23.0 ± 0.9**	—
Silybin A (**5**)	0.0 ± 2.3	8.4 ± 0.5**	10.3 ± 1.0**	15.4 ± 0.9**	24.2 ± 0.9**	27.6 ± 0.7**	—
Silybin B (**6**)	0.0 ± 1.1	5.7 ± 0.5	8.4 ± 0.8**	14.9 ± 0.2**	19.2 ± 2.1**	29.1 ± 3.0**	—
Isosilybin A (**7**)	0.0 ± 2.1	5.1 ± 2.9	7.0 ± 1.8	13.1 ± 2.6**	29.4 ± 1.9**	42.7 ± 2.7**	—
Isosilybin B (**8**)	0.0 ± 3.0	4.2 ± 3.5	4.8 ± 2.8	13.7 ± 2.8*	30.8 ± 2.4**	45.5 ± 3.2**	—
Luteolin (**15**)	0.0 ± 1.1	6.4 ± 1.0**	7.1 ± 0.8**	9.2 ± 0.5**	12.1 ± 0.7**	17.6 ± 1.4**	—
Kaempferol (**16**)	0.0 ± 0.8	5.7 ± 0.9**	7.6 ± 0.8**	12.1 ± 0.8**	24.2 ± 0.4**	38.4 ± 1.1**	—
Quercetin (**17**)	0.0 ± 0.9	3.5 ± 1.2*	4.8 ± 0.3**	14.7 ± 0.8**	45.8 ± 0.7**	—	ca. 31
3,4-Dihydroxybenzaldehyde (**36**)	0.0 ± 0.5	3.1 ± 1.9	2.3 ± 0.5	8.0 ± 1.2**	13.4 ± 0.3**	27.8 ± 1.4**	—
Coniferaldehyde (**44**)	0.0 ± 1.1	1.6 ± 0.6	4.2 ± 1.5*	7.3 ± 0.8**	13.9 ± 0.9**	18.6 ± 0.2**	—

	Concentration (μM)						IC_{50} (μM)
	0	10	30	100	300	1000	
Inhibition of tyrosinase (%)							
Kojic acid	0.0 ± 0.9	22.3 ± 2.1**	50.6 ± 0.6**	78.2 ± 0.7**	89.3 ± 0.3**	94.0 ± 0.2**	30

The incubation mixture of 70 μL of L-DOPA (2.5 mM in the phosphate buffer) as a substrate, and 20 μL of the test compound solution was prepared. The reaction was initiated by the addition of 120 μL of tyrosinase (80.5 units/mL) at 25 °C for 5 min, and then the absorbance at 405 nm (reference: 655 nm) was measured.
Each value represents the mean ± S.E.M. ($n = 4$).
Significantly different from the control group, $^{*}p < 0.05$, $^{**}p < 0.01$.
[a] The inhibitions by **2**, **10**, **19**, **20**, **21**–**24**, **29**, and **41** at 100 μM were less than 10.4%.

in melanin synthesis.[62] In the present study, to clarify the mechanism of action of isosilybin B (**8**), which showed the most potent activity, and related compounds (**5**–**7**), we examined the effects of **5**–**8** on the mRNA expression of tyrosinase and TRP-1 and -2 in the B16 melanoma 4A5 cells. As a result, **7** and **8** inhibited mRNA expression of TRP-2 at 3–30 μM. On the other hand, silybin B (**6**) also inhibited the mRNA expression of tyrosinase at a higher concentration (30 μM), but silybins A (**5**) and B (**6**) oppositely enhanced the mRNA expression of tyrosinase and TRP-1 and -2 mRNA at 10 and/or 30 μM (Table 4). The results in this and previous sections suggest that the inhibition of melanogenesis by **5** involves inhibition of enzyme activity of tyrosinase, but the enhanced expression of tyrosinase and TRP-1 and -2 reduce the melanogenesis. While, the inhibition of enzyme activity of tyrosinase and suppression of TRP-2 expression are involved in the inhibition of melanogenesis by **7** and **8**.

Recently, microphthalmia-associated transcriptional factor (MITF) has been shown to play an important role in melanogenesis,[25,63–66] and MITF phosphorylation by extracellular signal-regulated kinases (ERK1/2) suggested to be leading to an increase in melanogenesis.[25] On the other hand, ERKs-dependent phosphorylation of MITF is essential for MITF ubiquitinilation and its degradation. Previously, silibinin, a mixture of silybins and isosilybins, was reported to inhibit Raf/MEK/ERK pathway in MCF-7 human breast cancer cells.[67] Therefore, we examined effects of silybins A (**5**) and B (**6**) on phosphorylation of ERK1/2. As a result,

Table 4
Effects of compounds **5**–**8** and PD98059 on expression of tyrosinase and TRP-1 and-2 mRNA in B16 melanoma 4A5 cells

	Concentration (μM)				
	0	1	3	10	30
Tyrosinase mRNA/β-actin mRNA					
Silybin A (**5**)	1.00 ± 0.05	0.92 ± 0.10	1.12 ± 0.11	1.93 ± 0.08**	2.11 ± 0.26**
Silybin B (**6**)	1.00 ± 0.04	0.89 ± 0.09	0.95 ± 0.10	1.27 ± 0.04*	0.59 ± 0.07**
Isosilybin A (**7**)	1.01 ± 0.06	0.73 ± 0.06*	0.97 ± 0.08	0.85 ± 0.02	0.56 ± 0.06**
Isosilybin B (**8**)	1.00 ± 0.04	0.79 ± 0.12	1.15 ± 0.13	1.33 ± 0.09	0.87 ± 0.08
PD98059	1.00 ± 0.05	1.01 ± 0.10	1.23 ± 0.11	1.61 ± 0.11**	1.79 ± 0.10**
TRP-1 mRNA/β-actin mRNA					
Silybin A (**5**)	1.00 ± 0.03	0.79 ± 0.10	0.89 ± 0.08	1.60 ± 0.01**	1.07 ± 0.11
Silybin B (**6**)	1.01 ± 0.06	0.88 ± 0.10	1.12 ± 0.17	1.35 ± 0.05**	0.73 ± 0.08
Isosilybin A (**7**)	1.02 ± 0.12	1.11 ± 0.16	1.26 ± 0.13	1.12 ± 0.04	0.60 ± 0.07*
Isosilybin B (**8**)	1.01 ± 0.08	1.22 ± 0.12	1.02 ± 0.12	1.14 ± 0.09	1.14 ± 0.14
PD98059	1.00 ± 0.02	0.92 ± 0.11	1.18 ± 0.09	1.32 ± 0.04	1.53 ± 0.16*
TRP-2 mRNA/β-actin mRNA					
Silybin A (**5**)	1.01 ± 0.07	0.95 ± 0.11	1.00 ± 0.15	2.00 ± 0.09**	1.60 ± 0.19*
Silybin B (**6**)	1.00 ± 0.03	0.78 ± 0.14	1.31 ± 0.16	1.69 ± 0.09**	1.03 ± 0.11
Isosilybin A (**7**)	1.00 ± 0.05	0.68 ± 0.07**	0.63 ± 0.07**	0.63 ± 0.06**	0.36 ± 0.03**
Isosilybin B (**8**)	1.01 ± 0.06	0.97 ± 0.09	0.70 ± 0.09*	0.74 ± 0.07*	0.64 ± 0.06
PD98059	1.00 ± 0.04	1.12 ± 0.13	1.20 ± 0.13	1.30 ± 0.09	1.69 ± 0.10**

The melanoma cells (1.0×10^5 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. The total RNA was reverse transcribed to cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). Then a real-time PCR was carried out on a MiniOpticon real-time machine (Bio-Rad) using an iQ SYBR™ Green Supermix Kit (Bio-Rad). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to β-actin mRNA.
Each value represents the mean ± S.E.M. ($n = 4$).
Significantly different from the control group, $^{*}p < 0.05$, $^{**}p < 0.01$.

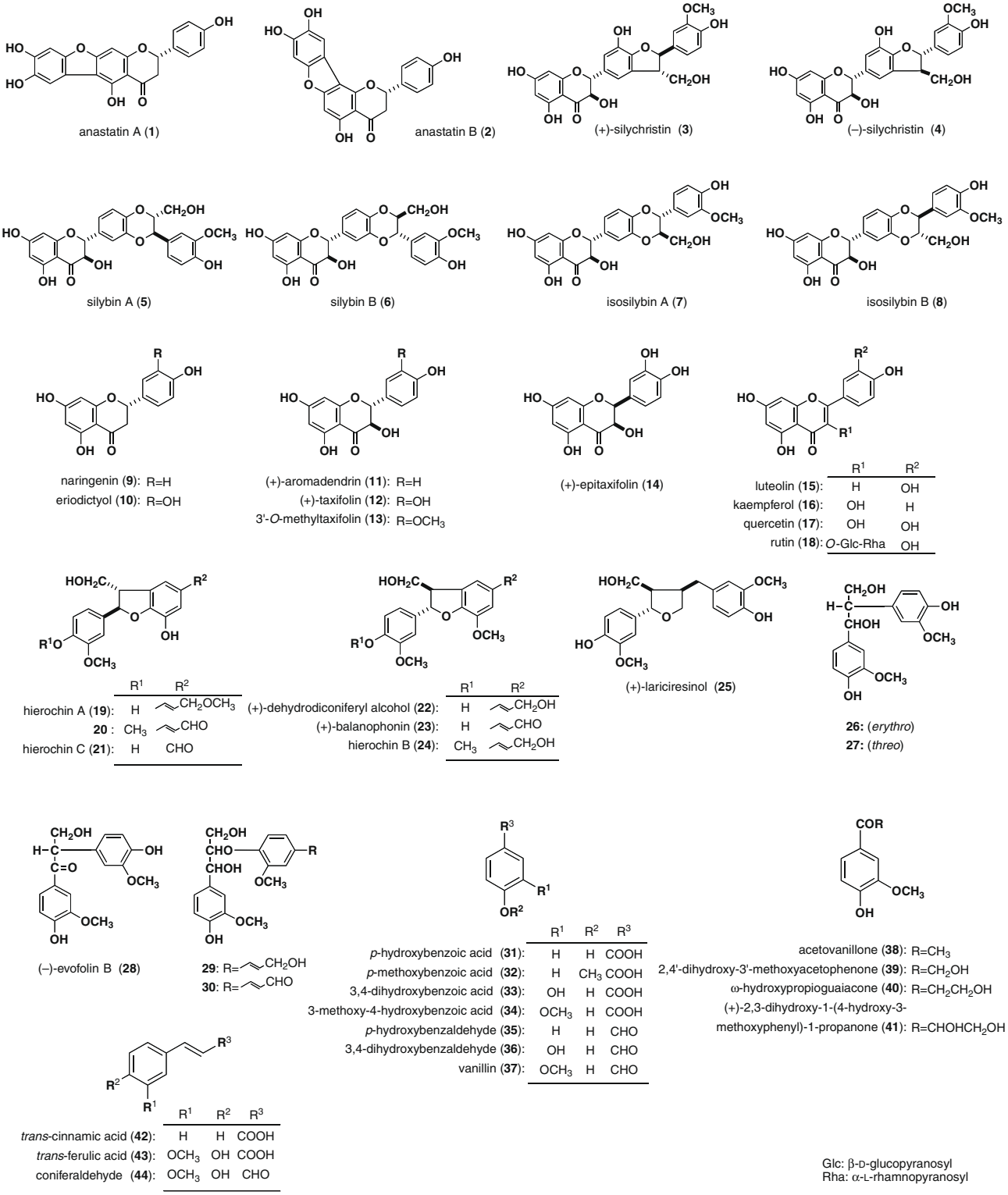

Figure 1. Chemical structures of **1**–**44** from *A. hierochuntica*.

PD98059, a well-known inhibitor of the phosphorylation of ERK1/2, inhibited the phosphorylation of ERK1/2 and enhanced the expression of tyrosinase, TRP-1 and -2 mRNA in a concentration-dependent manner (Fig. 2 and Table 4). Compound **6** inhibited the phosphorylation of ERK1/2 at 10–30 μM, but **5** inhibited it only at a higher concentration (100 μM). These findings suggest that the inhibition of ERK1/2 phosphorylation is partly involved in the enhanced expression of tyrosinase and TRP-1 and -2 mRNA by **5** and **6**, but their detailed mechanism of action should be studied further.

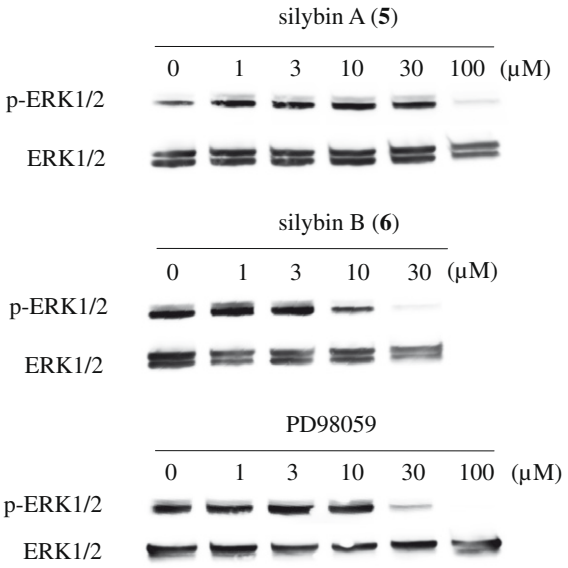

Figure 2. Effects of silybins A (**5**) and B (**6**) and PD98059 on phosphorylation of ERK1/2 in theophylline-stimulated B16 melanoma 4A5 cells. The melanoma cells (1.0×10^5 cells/2 mL/well) were seeded into 6-well multiplates and cultured for 24 h. The cells were then incubated with theophylline 1 mM and the test compound for 72 h. The ERK1/2 and phospho-ERK1/2 proteins were detected using SDS-PAGE Western blotting.

3. Conclusion

The methanolic extract of the whole plant of *A. hierochuntica* was found to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells. Among the constituents isolated, anastatin A (**1**), silybin A (**5**), isosilybins A (**7**) and B (**8**), eriodictyol (**10**), luteolin (**15**), kaempferol (**16**), quercetin (**17**), hierochins A (**19**) and B (**24**), **20**, (+)-dehydrodiconiferyl alcohol (**22**), (+)-balanophonin (**23**), **29**, and 3,4-dihydroxybenzaldehyde (**36**) substantially inhibited melanogenesis with IC_{50} values of 6.1–32 μM. With regard to the mechanism of action of **5**–**8**, the inhibition of tyrosinase activity suggested to be important. In addition, **7** and **8** inhibited the mRNA expression of TRP-2, but **5** and **6** oppositely enhanced the mRNA expression of tyrosinase, TRP-1 and TRP-2 at 10 and/or 30 μM, and the inhibition of phosphorylation of ERK1/2 is involved in the enhanced expression of mRNA, at least in part, similar to that of PD98059. The mechanism of action of the active constituents including their target molecules should be studied further.

4. Materials and methods

4.1. Extraction and isolation

The whole plants of *A. hierochuntica* (3.5 kg, collected in Egypt) were finely cut and extracted with MeOH three times under reflux for 3 h to give methanolic extract (184 g, 5.2%). The MeOH ext. (153 g) was suspended in water and then extracted with EtOAc and *n*-BuOH successively to give EtOAc (70 g, 2.4%), *n*-BuOH (18 g, 0.6%), and H_2O-soluble fractions (63 g, 2.2%), respectively. The EtOAc-soluble fraction (55 g) was subjected to ordinary-phase silica gel column chromatography [BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh) 1.65 kg, *n*-hexane–EtOAc (20:1→10:1→5:1→2:1→1:1, v/v)→$CHCl_3$–MeOH–H_2O (10:3:1, lower layer, v/v/v)→MeOH] to give seven fractions: Fr. 1 (1.65 g, 0.072%), Fr. 2 (1.21 g, 0.053%), Fr. 3 (31.4 g, 1.37%), Fr. 4 (1.38 g, 0.060%), Fr. 5 (1.68 g, 0.073%), Fr. 6 (1.86 g, 0.081%), and Fr. 7 (14.17 g, 0.62%). Fr. 4 (1.38 g) was subjected to reversed-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh) 40 g, MeOH–H_2O (45:55→75:25, v/v)→M!eOH] to give three fractions: Fr. 4–1 (171.7 mg), Fr. 4–2 (227.1 mg), and Fr. 4–3 (844.9 mg). Fraction 4–1 (171.7 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (35:65, v/v)] to give *p*-hydroxybenzoic acid (**31**, 4.6 mg, 0.00020%), *p*-hydroxybenzaldehyde (**35**, 21.2 mg, 0.00093%), vanillin (**37**, 81.8 mg, 0.0036%), and acetovanillone (**38**, 15.0 mg, 0.00066%). Fr. 4–2 (227.1 mg) was further purified by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (50:50, v/v)] to give *p*-methoxybenzoic acid (**32**, 17.2 mg, 0.00075%), *trans*-cinnamic acid (**42**, 13.5 mg, 0.00059%), and coniferaldehyde (**44**, 28.9 mg, 0.0013%). Fr. 5 (1.68 g) was subjected to reversed-phase silica gel column chromatography [50 g, MeOH–H_2O (20:80→45:55→60:40, v/v)→MeOH] to give six fractions: Fr. 5–1 (62.0 mg), Fr. 5–2 (313.9 mg), Fr. 5–3 (174.5 mg), Fr. 5–4 (298.0 mg), Fr. 5–5 (225.4 mg), and Fr. 5–6 (570.5 mg). Fr. 5–2 (313.9 mg) was separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (30:70, v/v)] to give *p*-hydroxybenzoic acid (**31**, 21.8 mg, 0.0010%), 3,4-dihydroxybenzoic acid (**33**, 18.7 mg, 0.00081%), 3-methoxy-4-hydroxybenzoic acid (**34**, 155.8 mg, 0.0068%), 3,4-dihydroxybenzaldehyde (**36**, 36.3 mg, 0.0016%), and 2,4′-dihydroxy-3′-methoxyacetophenone (**39**, 24.8 mg, 0.0011%). Fr. 5–3 (174.5 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (45:55, v/v)] to give *trans*-ferulic acid (**43**, 18.0 mg, 0.00079%), (+)-aromadendrin (**11**, 18.6 mg, 0.00081%), and 3′-*O*-methyltaxifolin (**13**, 8.6 mg, 0.00038%). Fr. 5–4 (298.0 mg) was separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (50:50, v/v)] to give naringenin (**9**, 87.0 mg, 0.0038%) and eriodictyol (**10**, 62.2 mg, 0.0027%). Fr. 5–6 (570.5 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (70:30, v/v)] to give anastatins A (**1**, 23.9 mg, 0.0010%) and B (**2**, 22.5 mg, 0.00098%). Fraction 6 (1.86 g) was subjected to reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (30:70→4!5:55→60:40, v/v)→MeOH] to afford five fractions: Fr. 6–1 (112.7 mg), Fr. 6–2 (1.19 g), Fr. 6–3 (72.1 mg), Fr. 6–4 (150.9 mg), and Fr. 6–5 (345.8 mg). Fr. 6–1 (112.7 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (20:80, v/v)] to give 3,4-dihydroxybenzoic acid (**33**, 39.0 mg, 0.0017%). Fr. 6–2 (1.19 g) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (35:65, v/v)] to give (+)-taxifolin (**12**, 1012.8 mg, 0.044%) and (+)-epitaxifolin (**14**, 79.8 mg, 0.0035%). Fr. 6–4 (150.9 mg) was separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (50:50, v/v)] to give quercetin (**17**, 22.9 mg, 0.0010%). Fraction 7 (14.17 g) was subjected to reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (30:70→50:50 →70:30→85:15, v/v)→MeOH] to give eight fractions: Fr. 7–1 (1.37 g), Fr. 7–2 (1.51 g), Fr. 7–3 (2.26 g), Fr. 7–4 (1.44 g), Fr. 7–5 (1.38 g), Fr. 7–6 (2.14 g), Fr. 7–7 (1.53 g), and Fr. 7–8 (2.46 g). Fr. 7–1 (1.37 g) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (20:80, v/v)] to give (+)-1,2-bis-(4-hydroxy-3-methoxyphenyl)-propane-1,3-diols [*erythro* form (**26**, 67.0 mg, 0.0029%) and *threo* form (**27**, 25.1 mg, 0.0011%)], and (+)-2,3-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (**41**, 34.2 mg, 0.0015%). Fr. 7–2 (1.51 g) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (30:70, v/v)] to give (−)-evofolin B (**28**, 21.3 mg, 0.00093%) and ω-hydroxypropioguaiacone (**40**, 35.0 mg, 0.0015%). Fr. 7–3 (2.26 g) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (45:55, v/v)] to give (+)-silychristin (**3**, 24.3 mg, 0.0011%), (−)-silychristin (**4**, 16.7 mg, 0.00073%), **20** (139.6 mg, 0.0061%), (+)-dehydrodiconiferyl alcohol (**22**, 24.5 mg, 0.0011%), (+)-balanophonin (**23**, 10.2 mg, 0.00045%), and **30** (5.4 mg, 0.00019%). Fr. 7–6 (2.14 g) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, CH_3CN–H_2O (30:70, v/v) and MeOH–H_2O (50:50, v/v)] to give silybin A (**5**, 24.9 mg,

0.0011%), silybin B (**6**, 31.7 mg, 0.0014%), isosilybin A (**7**, 34.1 mg, 0.0015%), and isosilybin B (**8**, 20.4 mg, 0.00090%).

The *n*-BuOH-soluble fraction (18 g) was subjected to ordinary-phase silica gel column chromatography [540 g, *n*-hexane–EtOAc (2:1→1:1, v/v)→$CHCl_3$–MeOH–H_2O (10:3:1→7:3:1, lower layer, v/v/v)→MeOH] to give six fractions: Fr. 1 (1.31 g, 0.045%), Fr. 2 (2.15 g, 0.073%), Fr. 3 (3.18 g, 0.109%), Fr. 4 (2.97 g, 0.101%), Fr. 5 (2.73 g, 0.093%), and Fr. 6 (5.75 g, 0.196%). Fr. 3 (3.18 g) was subjected to reversed-phase silica gel column chromatography [100 g, MeOH–H_2O (20:80→40:60→60:40, v/v)→MeOH] to give five fractions: Fr. 3–1 (835.5 mg), Fr. 3–2 (270.2 mg), Fr. 3–3 (418.4 mg), Fr. 3–4 (366.0 mg), and Fr. 3–5 (1.28 g). Fr. 3–3 (418.4 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, CH_3CN–H_2O (25:75, v/v)] to give hierochins B (**24**, 8.6 mg, 0.00029%) and C (**21**, 20.5 mg, 0.00070%). Fr. 3–4 (366.0 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, CH_3CN–H_2O (30:70, v/v)] to give hierochins A (**19**, 10.6 mg, 0.00046%) and D (22.1 mg, 0.0010%), and **29** (16.3 mg, 0.00056%). Fr. 4 (2.97 g) was subjected to reversed-phase silica gel column chromatography [90 g, MeOH–H_2O (30:70→0:50→70:30, v/v)→MeOH] and then separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (45:55, v/v) or CH_3CN–H_2O (25:75, v/v)] to give luteolin (**15**, 23.1 mg, 0.00078%) and (+)-lariciresinol (**25**, 71.3 mg, 0.0024%). Fr. 5 (2.73 g) was subjected to reversed-phase silica gel column chromatography [80 g, MeOH–H_2O (25:75→45:55 →75:25, v/v)→MeOH] to give five fractions: Fr. 5–1 (736.6 mg), Fr. 5–2 (285.2 mg), Fr. 5–3 (338.7 mg), Fr. 5–4 (455.1 mg), and Fr. 5–5 (896.0 mg). Fr. 5–2 (285.2 mg) was separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (20:80, v/v)] to give rutin (**18**, 21.2 mg, 0.00072%). Fr. 5–3 (338.7 mg) was further separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (45:55, v/v)] to give kaempferol (**16**, 17.6 mg, 0.00060%). Fr. 5–4 (455.1 mg) was also separated by HPLC [YMC-Pack ODS-A, 20 × 250 mm, id, MeOH–H_2O (95:5, v/v)] to give β-sitosterol 3-*O*-β-D-glucopyranoside (72.1 mg, 0.0025%). These known compounds were identified by comparison of their physical data ($[\alpha]_D$, ^{1}H NMR, ^{13}C NMR, MS) with reported values or authentic samples.[7,8,27–54]

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); Soluene®-350 was from Perkin Elmer (Waltham, MA, USA); RNeasy™ Mini Kit was from Qiagen (Huntsville, AL, USA); iScript™ cDNA Synthesis kit and iQ™ SYBR® Green Supermix were from Bio-Rad Laboratories (Hercules, CA, USA); the Cell Counting Kit-8™ was from Dojindo Laboratories (Kumamoto, Japan); anti-rabbit IgG, and horseradish peroxidase (HRP)-linked whole antibody from donkeys were from GE Healthcare (Buckinghamshire, UK); Blocking One and Blocking One-P 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-ERK1/2 and anti-phospho-ERK1/2 were from Cell Signaling Technology Japan, K.K. (Tokyo, Japan); 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.

4.2.3. Cell viability

The cell viability was assessed by our previous report[22] with a slight modification. The melanoma cells (5.0×10^3 cells/100 μL/well) were seeded into 96-well microplates and incubated for 24 h. After 70-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 2 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 Laboratories) 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.

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

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

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

4.2.4. Melanogenesis

Screening test for melanogensis using B16 melanoma 4A5 cells was performed as described previously[22] with slight modifications. The melanoma cells (2.0×10^4 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 Laboratories) at 405 nm (reference: 655 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.

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

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 tyrosinase activity was determined as described previously[22] with a slight modification. Briefly, the incubation mixture contained 70 μL of L-DOPA (2.5 mM in the phosphate buffer) as a substrate, and 20 μL of the test compound solution was prepared. The reaction was initiated by the addition of 120 μL of tyrosinase (80.5 units/mL) at 25 °C for 5 min, and then the absorbance at 405 nm (reference: 655 nm) was measured with a microplate reader (model 550, Bio-Rad Laboratories). 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.

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

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

4.2.6. Production of melanin from dopachrome by autoxidation

The autoxidation of dopachrome was performed according to the previous reports[60,61] with a slight modification. The tyrosinase solution (135 units/mL in PBS, 125 μL) was incubated at 25 °C for 10 min, and then dopa solution (0.03% in PBS, 125 μL) was added, and the mixture was incubated for 5 min. The test compound solution (125 μL) was then added to the solution. After 60 min, the reaction was stopped by the addition of 1 M HCl (50 μL) and the mixture was centrifuged at 10,000*g* for 15 min. The precipitates obtained were washed with 250 μL 6 M HCl and 500 μL distilled water successively, and dissolved in 500 μL Soluene®-350 (Perkin Elmer). The optical density at 400 nm was measured (model DU530, Beckman). The amount of melanin was determined based on the absorbance. The test compound was dissolved in DMSO, and the final concentration of DMSO in the PBS was 0.1%. Kojic acid was used as a reference compound.[60] The following equation was used to calculate the inhibition of production of melanin from dopachrome by autoxidation.

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

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

4.2.7. Expression of tyrosinase, TRP-1, and TRP-2 mRNA

The melanoma cells (1.0×10^5 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 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. cDNAs were synthesized from 1 μg total RNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Template cDNA thus obtained was incubated with gene-specific primers and with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) in a MiniOpticon (Bio-Rad Laboratories). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to β-actin mRNA. The thermal cycling program had initial denaturation (95 °C for 2 min) and then 40 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). The primer pairs were: tyrosinase primers, 5′-CAGATCTCTGATGGCCAT-3′ and 5′-GGATGACATAGACTGAGC-3′; TRP-1, 5′-CTTTCTCCCTTCCTTACTGG-3′; 5′-TGGCTTCATTCTTGGTGCTT-3′; TRP-2, 5′-TGAGAAGAAACAAAGTAGGCAGAA-3′ and 5′-CAACCCCAAGAGCAAGACGAAAGC-3′; and β-actin primers, 5′-ATGGGTCAGAAGGACTCCTACG-3′ and 5′-AGTGGTACGACCAGAGGCATAC-3′.

4.2.8. Immunoblotting

The melanoma cells (1.0×10^5 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 72 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 (20 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 or Blocking One-P (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-phospho-ERK1/2 (1:1000) and anti-ERK1/2 (1:1000) (Cell Signaling Technology Japan); anti-rabbit IgG; and HRP-linked whole antibody from donkeys (1:5000) (GE Healthcare).

4.2.9. Statistical analyzes

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

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References and notes

1. Rizk, A. M.; Hammouda, F. M.; Ismail, S. I.; Hassan, N. M.; Ahmed, F. A. *Int. J. Pharmacognosy* **1993**, *31*, 327.
2. Abou-Mandour, A. A.; Hartung, W. *Plant Cell Reports* **1995**, *14*, 657.
3. Murakami, T.; Kishi, A.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2000**, *48*, 994.
4. Yoshikawa, M.; Murakami, T.; Kishi, A.; Kagerura, T.; Matsuda, H. *Chem. Pharm. Bull.* **2001**, *49*, 863.
5. Murakami, T.; Kishi, A.; Yoshikawa, M. *Chem. Pharm. Bull.* **2001**, *49*, 974.
6. Morikawa, T.; Xu, F.; Matsuda, H.; Yoshikawa, M. *Heterocycles* **2002**, *57*, 1983.
7. Yoshikawa, M.; Xu, F.; Morikawa, T.; Ninomiya, K.; Matsuda, H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1045.
8. Yoshikawa, M.; Morikawa, T.; Xu, F.; Ando, S.; Matsuda, H. *Heterocycles* **2003**, *60*, 1787.
9. Xu, F.; Morikawa, T.; Matsuda, H.; Ninomiya, K.; Yoshikawa, M. *J. Nat. Prod.* **2004**, *67*, 569.
10. Abdel-Halim, O. B.; Morikawa, T.; Ando, S.; Matsuda, H.; Yoshikawa, M. *J. Nat. Prod.* **2004**, *67*, 1119.
11. Morikawa, T.; Abdel-Halim, O. B.; Matsuda, H.; Ando, S.; Muraoka, O.; Yoshikawa, M. *Tetrahedron* **2006**, *62*, 6435.
12. Morikawa, T.; Xu, F.; Kashima, Y.; Matsuda, H.; Ninomiya, K.; Yoshikawa, M. *Org. Lett.* **2004**, *6*, 869.
13. Morikawa, T.; Xu, F.; Ninomiya, K.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2004**, *52*, 494.
14. Hearing, V. J.; Körner, A. M.; Pawelek, J. M. *J. Invest. Dermatol.* **1982**, *79*, 16.
15. Hearing, V. J.; Jimenez, M. *Int. J. Biochem.* **1987**, *19*, 1141.
16. Prota, G. *Med. Res. Rev.* **1988**, *8*, 525.
17. Kuzumaki, T.; Matsuda, A.; Wakamatsu, K.; Ito, S.; Ishikawa, K. *Exp. Cell Res.* **1993**, *207*, 33.
18. Mishima, Y. *Pigment Cell Res.* **1994**, *7*, 376.
19. 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.
20. Pérez-Bernal, A.; Muñoz-Pérez, M. A.; Camacho, F. *Am. J. Clin. Dermatol.* **2000**, *1*, 261.
21. Parvez, S.; Kang, M.; Chung, H. S.; Cho, C.; Hong, M. C.; Shin, M. K.; Bae, H. *Phytother. Res.* **2006**, *20*, 921.
22. Matsuda, H.; Nakashima, S.; Oda, Y.; Nakamura, S.; Yoshikawa, M. *Bioorg. Med. Chem.* **2009**, *17*, 6048.
23. Friedmann, P. S.; Gilchrest, B. A. *J. Cell Physiol.* **1987**, *133*, 88.
24. Hunt, G.; Todd, C.; Cresswell, J. E.; Thody, A. J. *J. Cell Sci.* **1994**, *107*, 205.
25. Buscá, R.; Ballotti, R. *Pigment Cell Res.* **2000**, *13*, 60.
26. Steinberg, M. L.; Whittaker, J. R. *J. Cell Physiol.* **1976**, *87*, 265.
27. Hansel, R.; Kaloga, M. *Tetrahedron Lett.* **1977**, *51*, 4547.
28. Lee, D. Y. W.; Liu, Y. *J. Nat. Prod.* **2003**, *66*, 1171.
29. Niwa, M.; Otsuji, S.; Tatematsu, H.; Liu, G.; Chen, X.; Hirata, Y. *Chem. Pharm. Bull.* **1986**, *34*, 3249.
30. Matsuda, H.; Morikawa, T.; Toguchida, I.; Harima, S.; Yoshikawa, M. *Chem. Pharm. Bull.* **2002**, *50*, 972.
31. Bellakhdar, J.; Passannanti, S.; Paternostro, M. P.; Piozzi, F. *Planta Med.* **1988**, *54*, 94.
32. Nonaka, G.; Goto, Y.; Kinjo, J.; Nohara, T.; Nishioka, I. *Chem. Pharm. Bull.* **1987**, *35*, 1105.
33. Macro, J. A.; Sanz-Cervera, J. F.; Yuste, A.; Oriola, M. C. *Phytochemistry* **1994**, *36*, 725.
34. Luteolin (**15**), kaempferol (**16**), quercetin (**17**), and rutin (**18**), and *p*-hydroxybenzaldehyde (**35**) were identified by comparison of their physical data (IR, UV, MS, and ^{1}H NMR and ^{13}C NMR) with those of authentic samples.
35. Greca, M. D.; Molinaro, A.; Monaco, P.; Previtera, L. *Heterocycles* **1994**, *38*, 1099.
36. Takehara, T.; Sasaya, T. *Mokuzai Gakkaishi* **1979**, *25*, 660.

37. Hirai, N.; Okamoto, M.; Udagawa, H.; Yamamuro, M.; Kato, M.; Koshimizu, K. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1679.
38. Yuen, M. S. M.; Xue, F.; Mak, T. C. W.; Wong, H. N. C. *Tetrahedron* **1998**, *54*, 12429.
39. Duh, C. Y.; Phoebe, C. H.; Pezzuto, J.; Kinghorn, A. D.; Farnsworth, N. R. *J. Nat. Prod.* **1986**, *49*, 706.
40. Miki, K.; Takehara, T.; Sasaya, T.; Sakakibara, A. *Phytochemistry* **1980**, *19*, 449.
41. Yoshikawa, K.; Mimura, N.; Arihara, S. *J. Nat. Prod.* **1998**, *61*, 1137.
42. Wu, T.; Yeh, J. H.; Wu, P. *Phytochemistry* **1995**, *40*, 121.
43. Li, S.; Lundquist, K.; Wallis, A. F. A. *Phytochemistry* **1998**, *49*, 2125.
44. Scott, K. N. *J. Am. Chem. Soc.* **1972**, *94*, 8564.
45. Dawn, M.; Edwin, C. W.; Burt, Z. *Phytochemistry* **1969**, *8*, 393.
46. Cordores, C. G.; Bartolome, B.; Jimeno, M. L. *J. Agric. Food Chem.* **1997**, *45*, 873.
47. Muhlenbeck, U.; Kortenbusch, A.; Barz, W. *Phytochemistry* **1996**, *42*, 1573.
48. Achenbach, H.; Stocker, M.; Constenla, M. A. *Phytochemistry* **1988**, *27*, 1835.
49. Baderschneider, B.; Winterhalter, P. *J. Agric. Food Chem.* **2001**, *49*, 2788.
50. Satake, T.; Murakami, T.; Saiki, Y.; Chen, C.; Gomez, P. L. D. *Chem. Pharm. Bull.* **1984**, *32*, 4620.
51. Kosuge, K.; Mitsnaga, K.; Koike, K.; Ohmoto, T. *Chem. Pharm. Bull.* **1994**, *42*, 1669.
52. Boonyaratavej, S.; Tantayanontha, S.; Kitchauachai, P.; Chaichantipyuth, C.; Chittawong, V.; Miles, D. H. *J. Nat. Prod.* **1992**, *55*, 1761.
53. Sy, L. K.; Brown, G. D. *Phytochemistry* **1999**, *50*, 781.
54. Kojima, H.; Sato, N.; Hatano, A.; Ogura, H. *Phytochemistry* **1990**, *29*, 2351.
55. Choi, M. Y.; Song, H. S.; Hur, H. S.; Sim, S. S. *Arch. Pharm. Res.* **2008**, *31*, 1166.
56. Fuji, T.; Saito, M. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1989.
57. Kubo, I.; Nitoda, T.; Nihei, K. *Molecules* **2007**, *12*, 1045.
58. Nitoda, T.; Isobe, T.; Kubo, I. *Phytother. Res.* **2008**, *22*, 867.
59. 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.
60. Mason, H. S.; Peterson, E. W. *Biochim. Biophys. Acta* **1965**, *111*, 134.
61. Matsuda, H.; Higashino, M.; Nakai, Y.; Iinuma, M.; Kubo, M.; Lang, F. A. *Biol. Pharm. Bull.* **1996**, *19*, 153.
62. Bertolotto, C.; Busca, R.; Abbe, P.; Bille, K.; Aberdam, E.; Ortonne, J. P.; Ballotti, R. *Mol. Cell Biol.* **1998**, *18*, 694.
63. Tachibana, M. *J. Invest. Dermatol. Symp. Proc.* **2001**, *6*, 95.
64. Levy, C.; Khaled, M.; Fisher, D. E. *Trends Mol. Med.* **2006**, *12*, 406.
65. Kim, Y. J.; No, J. K.; Lee, J. S.; Kim, M. S.; Chung, H. Y. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 532.
66. Maeda, K.; Naitou, T.; Umishio, K.; Fukuhara, T.; Motoyama, A. *Biol. Pharm. Bull.* **2007**, *30*, 873.
67. Kim, S.; Choi, J. H.; Lim, H. I.; Lee, S. K.; Kim, W. W.; Kim, J. S.; Kim, J. H.; Choe, J. H.; Yang, J. H.; Nam, S. J.; Lee, J. E. *Phytomedicine* **2009**, *16*, 573.