Free-Radical-Scavenging, Antityrosinase, and Cellular Melanogenesis Inhibitory Activities of Synthetic Isoflavones

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In this study, we examined the potential of synthetic isoflavones for application in cosmeceuticals. Twenty-five isoflavones were synthesized and their capacities of free-radical-scavenging and mushroom tyrosinase inhibition, as well as their impact on cell viability of B16F10 murine melanoma cells and HaCaT human keratinocytes were evaluated. Isoflavones that showed significant mushroom tyrosinase inhibitory activities were further studied on reduction of cellular melanin formation and antityrosinase activities in B16F10 melanocytes *in vitro*. Among the isoflavones tested, 6-hydroxydaidzein (**2**) was the strongest scavenger of both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals with SC_{50} values of 11.3 ± 0.3 and 9.4 ± 0.1 μM, respectively. Texasin (**20**) exhibited the most potent inhibition of mushroom tyrosinase (IC_{50} 14.9 ± 4.5 μM), whereas retusin (**17**) showed the most efficient inhibition both of cellular melanin formation and antityrosinase activity in B16F10 melanocytes, respectively. In summary, both retusin (**17**) and texasin (**20**) exhibited potent free-radical-scavenging capacities as well as efficient inhibition of cellular melanogenesis, suggesting that they are valuable hit compounds with potential for advanced cosmeceutical development.

Introduction. – Excessive solar exposure usually causes the generation of cellular reactive oxygen species (ROS), which may lead to skin lesions including aging, wrinkles, melasma, hyperpigmentation, and tumors [1]. Although antioxidants have been widely applied both in skin protection and beautification, the increase of consumer demands has encouraged cosmetic producers to launch more research in the development of new cosmetic ingredients and formulations since the 1980's [2]. The major concerns of consumers about cosmetic formulations are the substantive efficacy and safety of the antioxidants or cosmeceuticals [2][3]. Many botanicals are considered as safer ingredients when applied in cosmetics [3][4]. Natural products currently used in commercial cosmetics such as arbutin and kojic acid are popular for their skin whitening properties, but they are deficient in antioxidant capacity. Therefore, the development of safer and more effective cosmeceuticals for skin care from botanicals continues to be an important research target [5].

Flavonoids, a family of well-known polyphenols, have long received a great deal of attention for use in the cosmeceutical development [4–7]. They are known to have numerous advantages when used as cosmetic ingredients: *i*) they are effective in blood vessel protection, antiplatelet aggregation, and capillary permeability decrease [6], *ii*)

numerous flavonoids have demonstrated powerful tyrosinase inhibitory activities, suggesting their potential in skin lightening applications [5], and *iii*) flavonoids are believed to be innocuous and pharmacologically beneficial botanicals that can satisfy the safety prerequisite in cosmeceutical applications [3][4][7].

Isoflavones represent a subfamily of the flavonoid. Genistein, the most distinguished isoflavone, is known as cardiovascular-protecting phytoestrogen. It can eradicate UV-induced skin aging [8], and its glucoside is also capable of inhibiting melanoma cell growth [9], but it lacks the tyrosinase inhibitory activity [10–12]. In addition, some *ortho*-dihydroxylated isoflavones obtained from fermented soy foodstuffs were shown to have notable pharmacological benefits in cosmeceutical applications [10–16]. For example, 7,8,4′-trihydroxyisoflavone (**1**) and 5,7,8,4′-tetrahydroxyisoflavone were suicide substrates of mushroom tyrosinase [10][13][14] and effective cellular melaninogenetic inhibitors [12][13], 6,7,4′-trihydroxyisoflavone (**2**) was reported to be a potent mushroom tyrosinase inhibitor [10][11], whereas 7,3′,4′-trihydroxyisoflavone showed potent inhibitory efficacy both on mushroom tyrosinase (EC 1.14.18.1) and on melanin formation of B16 melanocytes [12]. Calycosin, an isoflavone isolated from *Astragalus membranaceus*, also exhibited effective inhibition of melanin biosynthesis [16]. Furthermore, some of these isoflavones were reported to possess free-radical-scavenging activities [15][17][18].

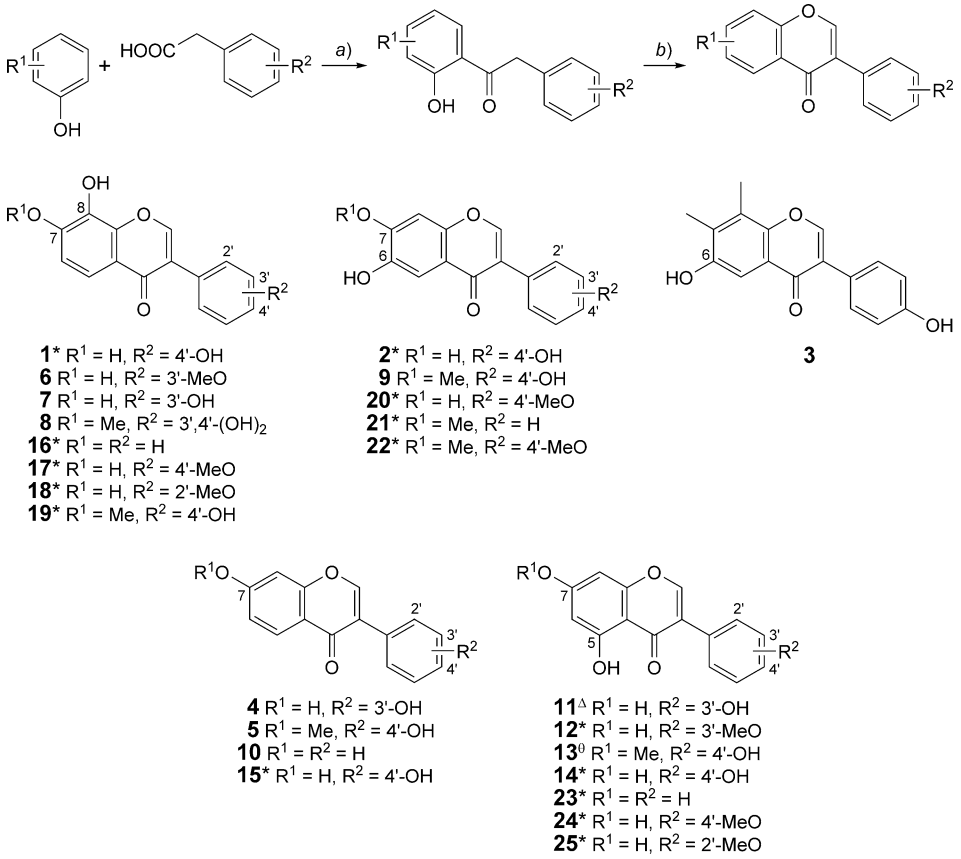
Our previous works have shown that dexoybenzoins, the cyclization precursors and potential metabolites of isoflavones [19][20], are potent antioxidants [19] and powerful porcine arterial relaxants [20]. In this study, we further synthesized a series of polyphenolic isoflavones and inspected their potential for cosmeceutical application. Twenty-five isoflavones were assayed on $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging, antityrosinase activity, and impact on cell viability of B16F10 murine melanoma cells and HaCaT human keratinocytes. Those isoflavones that showed significant mushroom tyrosinase inhibitory activities were also assayed on reduction of cellular melanin generation and tyrosinase inhibitory activity in B16F10 melanocytes *in vitro*.

Results and Discussion. – *Design and Synthesis of Isoflavones.* The reported one-pot reaction facilitated the synthesis of polyoxygenated isoflavones [21]. In general, a compound with one or two phenolic OH groups could be a suitable substrate for tyrosinase by mimicking the ring identity of its primary substrates, tyrosine and dopa [5]. Furthermore, compounds with more phenolic OH groups possibly have greater antioxidant capacities [19]. Structure–activity relationship (SAR) studies can be conducted to validate the biological potency contributed by various O-bearing substituents in isoflavones. Based on the reported approach [21], in the present work, 25 isoflavones were successfully synthesized, in which the O-bearing substituents on ring *A* were located at C(6), C(7), C(5) and at C(7), C(6) and at C(7), or at C(7) and C(8), combined with various substituents at ring *B* [21]. In addition, the O-bearing substituents on ring *B* were primarily focused at C(3′) and C(4′).

Isoflavones **3**–**9** were obtained by coupling of related phenols with various phenylacetic acids with $BF_3 \cdot Et_2O$ as catalyst, followed by cyclization with MeSO$_2$Cl–DMF complex (*Scheme*) [21]. Because 2-hydroxydeoxybenzoins are key intermediates in this synthesis, the major synthetic work in this study was the previously reported cyclization of deoxybenzoins [19][20]. Besides, 7-hydroxyisofla-

vone (**10**) was a product reported previously [22], isoflavone **11** was the demethylated product of mutabilein (**12**), which was heated under reflux in HBr (48%)/AcOH 1:1, and prunetin (**13**) was obtained by monomethylation of genistein (**14**). The isoflavones used in this study can be categorized according to the position of the O-bearing substituents on ring *A* as 6-oxygenated (*i.e.*, **3**), 7-oxygenated series (*i.e.*, **4**, **5**, **10**, and **15**), 7,8-dioxygenated series (*i.e.*, **1**, **6**–**8**, and **16**–**19**), 6,7-dioxygenated series (*i.e.*, **2**, **9**, and **20**–**22**), and 5,7-dioxygenated series (*i.e.*, **11**–**14** and **23**–**25**) with various substituents on ring *B* (*Scheme*). Isoflavones **5**, **8**, **9**, **13**, **19**, and **21** can also be alternatively classified as 7-methoxylated series. The 7-methoxylated series is used for comparing substitution–activity relationship with those isoflavones with a MeO group at C(4′) (*p*-anisyl ring *B*). Among the investigated isoflavones, **7**, **18**, and **19** were newly synthesized. Isoflavone **11** was already reported as a natural product, but NMR data were lacking [23]. Generally, the yields of synthetic isoflavones are satisfactory and

Scheme. *One-Pot Synthesis and Structures of Isoflavones* **1**–**25**



1* R^1 = H, R^2 = 4'-OH
6 R^1 = H, R^2 = 3'-MeO
7 R^1 = H, R^2 = 3'-OH
8 R^1 = Me, R^2 = 3',4'-$(OH)_2$
16* R^1 = R^2 = H
17* R^1 = H, R^2 = 4'-MeO
18* R^1 = H, R^2 = 2'-MeO
19* R^1 = Me, R^2 = 4'-OH

2* R^1 = H, R^2 = 4'-OH
9 R^1 = Me, R^2 = 4'-OH
20* R^1 = H, R^2 = 4'-MeO
21* R^1 = Me, R^2 = H
22* R^1 = Me, R^2 = 4'-MeO

3

4 R^1 = H, R^2 = 3'-OH
5 R^1 = Me, R^2 = 4'-OH
10 R^1 = R^2 = H
15* R^1 = H, R^2 = 4'-OH

11$^{\Delta}$ R^1 = H, R^2 = 3'-OH
12* R^1 = H, R^2 = 3'-MeO
13$^{\theta}$ R^1 = Me, R^2 = 4'-OH
14* R^1 = H, R^2 = 4'-OH
23* R^1 = R^2 = H
24* R^1 = H, R^2 = 4'-MeO
25* R^1 = H, R^2 = 2'-MeO

a) $BF_3 \cdot Et_2O$, 90°, 2 h, N_2. *b*) $BF_3 \cdot$DMF, $MeSO_2Cl$–DMF, 70°, 2 h. * Synthesized *via* process *b* from related deoxybenzoins reported previously [19][20]. Δ Demethylated product of **12**. θ Methylated product of **14**.

Table 1. *^{13}C-NMR Data* (100 MHz) *of Synthesized Isoflavones.* δ in ppm, J in Hz.

Compound	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)	C(10)	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	C(6′)	Me
1[a])	153.2	123.2	177.4	116.1	114.2	150.4	132.9	147.3	117.6	124.2	130.3	115.0	157.5	115.0	130.3	
2[b])	152.6	124.6[c])	176.0	109.8	146.9	154.7	104.1	152.6	118.5	124.6[c])	131.4	116.5	159.2	116.5	131.4	
3[b])	153.0	124.4[c])	176.8	106.0	154.7	132.8	127.5	149.3	124.2	124.4[c])	131.3	116.6	159.3	116.6	131.1	12.2, 13.3
4[a])	154.1	124.8	176.6	127.5	115.3	163.5	102.1	158.5	117.0	133.4	114.9	157.3	116.1	129.3	120.1	
5[b])	153.0	119.3	176.0	128.1	115.1	164.6	101.1	159.4	116.5	125.6	131.3	116.6	158.6	116.6	131.3	56.2
6[b])	153.3	124.3	175.9	116.7	115.5	152.2	134.7	147.9	118.8	134.9	113.8	160.0	115.3	129.6	121.9	55.1
7[b])	152.4	125.0	176.4	117.9	115.6	153.5	135.2	148.3	119.2	135.1	116.2	159.2	117.1	130.2	120.7	
8[b])	152.9	124.4	176.4	115.8	110.1	151.7	136.4	147.0	120.1	124.6	120.9	147.5	147.0	116.4	118.0	56.3
9[b])	152.8	124.7	176.1	109.8	147.2	154.8	100.7	152.0	119.4	124.3	131.3	116.5	159.3	116.5	131.3	55.5
11[d])	154.4	123.4	180.7	163.3	93.9	164.4	99.3	158.3	105.5	132.7	115.3	157.4	116.4	129.5	120.3	
12[d])	154.6	123.3	180.6	163.3	93.9	164.5	99.3	158.3	105.5	132.7	113.8	159.9	115.0	129.4	121.4	54.9
13[d])	154.1	122.4	181.7	163.5	92.9	166.2	99.0	158.6	107.0	124.2	131.3	116.7	159.7	116.7	131.3	56.2
14[b])	152.2	115.0	180.0	162.5	93.4	164.8	98.9	158.1	104.6	121.2	129.8	115.1	157.5	115.1	129.8	
15[a])	153.5	123.1	177.0	127.3	115.2	163.4	102.0	158.6	117.0	124.8	130.2	115.0	157.5	115.0	130.2	
16[d])	154.5	125.5	176.6	117.9	115.5	151.3	134.2	148.2	119.8	134.4	130.6	129.6	129.2	129.6	130.6	
17[d])	153.8	125.2	176.7	118.0	115.4	151.0	134.3	148.2	119.9	126.3	131.7	115.0	161.1	115.0	131.7	56.2
18[d])	155.0	123.5	176.2	117.9	115.3	151.0	134.4	148.3	119.9	123.2	159.4	112.7	133.4	121.6	131.0	56.6
19[b])	153.2	124.9[c])	176.8	116.2	110.7	152.1	136.9	147.5	120.6	124.9[c])	131.4	116.5	159.3	116.5	131.4	56.8
20[b])	152.8	126.2[c])	175.9	109.8	146.9	154.8	104.1	152.2	118.5	126.2[c])	131.1	114.5	160.1	114.5	131.1	55.5
21[b])	153.6	124.3	175.6	109.9	147.3	154.8	100.8	151.9	119.5	133.8	129.9	129.0	128.4	129.0	129.9	56.6
22[b])	153.0	124.3	175.8	109.9	147.2	154.8	100.7	152.0	119.5	126.0	131.1	114.5	160.2	114.5	131.1	55.5, 56.6
23[d])	154.5	123.5	180.7	163.3	93.9	164.5	99.3	158.4	105.5	131.5	129.3	128.4	128.2	128.4	129.3	
24[b])	153.6	123.3	181.0	163.6	94.6	166.1	100.2	158.6	105.7	123.9	130.8	114.3	160.1	114.3	130.8	55.2
25[d])	155.1	121.3	180.5	163.2	93.9	164.3	99.2	158.1	105.5	120.4	158.4	111.5	131.9	120.4	130.1	55.4

[a]) Detected in CD_3OD. [b]) Detected in (D_5)pyridine. [c]) Signals could be overlapped in each row. [d]) Detected in (D_6)acetone.

they can be easily obtained. *Table 1* shows the ^{13}C-NMR data of the synthesized isoflavones **1**–**9** and **11**–**25**.

Ingredients of commercial cosmetics with dual efficacy, antioxidant and antityrosinase activities, are rare; if they show both activities, the performance will be better in cosmeceutical applications. Accordingly, the evaluation assays of these isoflavones focused on free-radical-scavenging capacity as well as mushroom tyrosinase inhibitory effect. Artificial free radicals, such as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, are commonly used in antioxidant assays, which can provide a reliable antioxidant profile of tested compounds. The screening results of the synthesized isoflavones are presented below.

$ABTS^{\bullet+}$ Radical-Scavenging Activities. The $ABTS^{\bullet+}$ radical-scavenging results indicated that isoflavones with two OH groups in *ortho* position to each other (in **1**, **2**, **6**–**8**, **16**–**18**, and **20**), either on ring *A* (6,7-dihydroxy or 7,8-dihydroxy) or ring *B* (3′,4′-dihydroxy; in **8**), demonstrated potent $ABTS^{\bullet+}$ radical-scavenging activities as shown in *Table 2*. These isoflavones exhibited $ABTS^{\bullet+}$ SC_{50} values comparable to the positive control vitamin C (SC_{50} 19.1 ± 0.0 and 19.0 ± 0.1 μM). 6-Hydroxydaidzein (**2**) was the most active $ABTS^{\bullet+}$ scavenger, with SC_{50} values of 20.1 ± 1.4 and 11.3 ± 1.3 μM at two incubation intervals.

The *ortho*-dihydroxylated isoflavones **1**, **2**, **6**–**8**, **16**–**18**, and **20** showed the highest $ABTS^{\bullet+}$ radical-scavenging activities in this study as shown in *Table 2*. The acidic OH group at C(7) (or at C(4′) alternatively) could have a counterion effect after ionization, which may play an important role in $ABTS^{\bullet+}$ radical scavenging, and the effect was similar to that of the OH group at C(4) of deoxybenzoins [19]. The counterion effect may have facilitated the contact of the cationic free radical ($ABTS^{\bullet+}$) with the anionic scavengers [19]. The $ABTS^{\bullet+}$ radical-scavenging activities of *ortho*-dihydroxylated isoflavones were abolished thoroughly after *O*-methylation at C(7), which also terminated the counterion phenomenon, such as in the cases of **1** *vs.* **19** and **20** *vs.* **22**.

$DPPH^{\bullet}$ Radical-Scavenging Activities. The results indicated that *ortho*-dihydroxylated isoflavones (*i.e.*, **1**, **2**, **6**–**8**, and **16**–**20**) are usually potent $DPPH^{\bullet}$ radical scavengers (*Table 2*). Among these isoflavones, 6-hydroxydaidzein (**2**) exhibited the strongest $DPPH^{\bullet}$ radical-scavenging activity at two check intervals with SC_{50} values of 9.1 ± 0.0 and 9.4 ± 0.1 μM, which were three times stronger than for the positive control vitamin C (SC_{50} 28.6 ± 2.5 and 28.6 ± 1.7 μM). Retusin (**17**; SC_{50} 12.3 ± 0.6 and 11.8 ± 0.8 μM) and texasin (**20**; SC_{50} 13.7 ± 0.5 and 11.7 ± 0.4 μM) both showed nearly 2.5-fold higher $DPPH^{\bullet}$ radical-scavenging activities than the positive control. 8-Hydroxydaidzein (**1**; SC_{50} 16.5 ± 0.5 and 14.3 ± 1.1 μM), the isomer of 6-hydroxydaidzein (**2**), also exhibited a $DPPH^{\bullet}$ radical-scavenging activity of about twice the potency of vitamin C. Furthermore, the other *ortho*-dihydroxylated isoflavones including **6**–**8**, **16**, and **18** generally showed $DPPH^{\bullet}$ radical-scavenging activities comparable to that of vitamin C (*Table 2*). Briefly, the sequence in $DPPH^{\bullet}$ radical-scavenging potency based on the SC_{50} values was **2** > **20** ≥ **17** > **1** > **18** > **8** > **16** > **7** ≥ vitamin C ≥ **6**.

The obtained powerful $DPPH^{\bullet}$ radical scavengers including 8-hydroxydaidzein (**1**), 6-hydroxydaidzein (**2**), retusin (**17**), and texasin (**20**) suggested that the two OH groups in *ortho* position of an isoflavone play an important role in the activity. Although isoflavone **8**, which has an *ortho*-dihydroxylated ring *B*, also exhibited significant $DPPH^{\bullet}$ radical-scavenging activity, its potency was only about half of the former mentioned isoflavones. Thus, the free-radical-scavenging capability of an isoflavone

Table 2. SC_{50} *Values of Isoflavones on ABTS*$^{\cdot+}$ *and DPPH*$^{\cdot}$ *Free Radical Scavenging Activity, and* IC_{50} *Values on Mushroom Tyrosinase Inhibitory Assay*[a])

Compound	SC_{50} [μM]				IC_{50} [μM]	
	ABTS$^{\cdot+}$		DPPH$^{\cdot}$		Tyrosinase	
	7 min	20 min	30 min	60 min	30 min	60 min
1	24.9 ± 0.4	24.7 ± 0.6	16.5 ± 0.5	14.3 ± 1.1	45.0 ± 0.9	42.3 ± 6.6
2	20.1 ± 1.4	11.3 ± 1.3	9.1 ± 0.0	9.4 ± 0.1	15.6 ± 2.4	24.3 ± 4.2
3	ns[c])	ns	ns	ns	45.8 ± 1.8	52.3 ± 8.0
4	ns	ns	ns	ns	110.9 ± 10.2	129.2 ± 2.2
5	ns	ns	ns	ns	38.1 ± 8.6	54.4 ± 1.9
6	25.5 ± 0.4	25.2 ± 0.4	30.0 ± 1.2	29.3 ± 1.2	53.9 ± 11.9	54.2 ± 9.2
7	25.2 ± 0.0	24.9 ± 0.1	28.8 ± 0.3	28.3 ± 0.2	96.4 ± 6.3	100.2 ± 6.3
8	25.2 ± 1.8	23.2 ± 1.5	23.6 ± 1.1	20.3 ± 0.9	129.9 ± 2.3	146.8 ± 5.4
9	nd[d])	nd	nd	nd	83.5 ± 4.2	118.8 ± 7.8
10	ns	ns	ns	ns	94.6 ± 11.2	108.9 ± 6.4
11	ns	ns	ns	ns	181.3 ± 0.9	197.7 ± 4.9
12	ns	ns	ns	ns	180.4 ± 7.3	169.2 ± 10.0
13	nd	nd	nd	nd	113.9 ± 8.5	105.9 ± 11.9
14	nd	nd	nd	nd	ns	ns
15	nd	nd	nd	nd	ns	ns
16	28.4 ± 1.0	28.1 ± 0.8	24.2 ± 0.4	23.8 ± 0.5	76.1 ± 7.6	74.0 ± 9.2
17	26.3 ± 1.6	26.5 ± 1.7	12.3 ± 0.6	11.8 ± 0.8	50.9 ± 1.8	51.8 ± 4.6
18	26.1 ± 0.6	25.9 ± 0.9	17.5 ± 0.9	16.9 ± 1.0	82.8 ± 5.0	105.5 ± 10.7
19	ns	ns	113.0 ± 8.4	79.2 ± 5.6	77.2 ± 10.7	85.2 ± 7.4
20	44.3 ± 5.7	27.8 ± 0.0	13.7 ± 0.5	11.7 ± 0.4	8.1 ± 0.2	14.9 ± 4.5
21	nd	nd	ns	ns	145.9 ± 19.6	199.9 ± 22.8
22	ns	ns	ns	ns	141.1 ± 4.4	154.3 ± 19.3
23	ns	ns	ns	ns	170.0 ± 10.4	199.6 ± 15.9
24	ns	ns	ns	ns	141.3 ± 4.7	166.0 ± 24.0
25	ns	ns	ns	ns	171.1 ± 4.2	177.7 ± 8.2
Vitamin C[e])	19.1 ± 0.0	19.0 ± 0.1	28.6 ± 2.5	28.6 ± 1.7	–	–
Arbutin[f])	–	–	–	–	154.0 ± 4.9	146.2 ± 8.3

[a]) Data are presented as mean ± SD ($n \geq 4$). [b]) SC_{50}, concentration of 50% free radical scavenged. [c]) ns, no significance ($IC_{50} > 300$ μM). [d]) nd, not detected due to poor solubility (for **9**, **13**, and **21**) or neglected, such as genistein (**14**) and daidzein (**15**). [e]) Vitamin C (ascorbic acid) was used as positive control of radical scavengers. [f]) Arbutin, a commercial whitening agent, was used as positive control in the anti-tyrosinase assay.

seems to be better when the two OH groups in *ortho* position are located on ring *A*. In addition, the free OH group at C(7) that keeps the two *ortho*-positioned OH groups of ring *A* intact can be crucial for the free-radical-scavenging activity of an isoflavone; for example, the free-radical-scavenging activities of both **1** and **20** disappeared when the OH group at C(7) was methylated (in **19** and **22**). According to the scaffold of an isoflavone, the OH groups at C(7) and C(4′) are both acidic due to the electron-withdrawing effect of γ-pyronic ring *C*. In other words, the ring *C* system of an isoflavone will keep the electrons of the acidic OH group from being taken away by a radical but the *ortho* OH group can disperse the electron-withdrawing effect and donate enough electrons for radical scavenging. The electron-donating property of the

ortho OH group can stabilize the isoflavone C(7)-aroxyl radical formed after one electron is lost [24]. Furthermore, the isoflavone aroxyl radical can endure an additional electron loss to scavenge another free radical [24]; this explains why the *ortho*-dihydroxylated series (*i.e.*, **1**, **2**, **6**–**8**, **16**–**18**, and **20**) exhibited stronger free-radical-scavenging activities. In brief, the most suitable skeleton to show powerful radical-scavenging activity of an isoflavone could be an *ortho*-dihydroxylated ring *A* combined with an electron-rich ring *B* that has an electron-donating substituent, such as OH or MeO, in *para* position.

Mushroom Tyrosinase Inhibitory Assay. The mushroom tyrosinase (EC 1.14.18.1) inhibitory assay is a commonly used technique for preliminary screening of skin-whitening agents. *Table 2* shows that besides genistein (**14**) and daidzein (**15**) all other synthetic isoflavones appeared to have antityrosinase activities, with IC_{50} values varying from 14.9 ± 4.5 (**20**) to 199.9 ± 22.8 μM (**21**). The order of potency for isoflavones exhibiting antityrosinase activities stronger than that of the positive control arbutin (IC_{50} 154.0 ± 4.9 and 146.2 ± 8.3 μM) was texasin (**20**) > 6-hydroxydaidzein (**2**) > 8-hydroxydaidzein (**1**) > retusin (**17**) ≥ **3** > **6** ≥ isoformononetin (**5**) > **16** > **19** > **7** > **18** ≥ **13** > **10** > **9** > **4** > arbutin. Texasin (**20**), 6,7-dihydroxy-4′-methoxyisoflavone, exhibited the most potent mushroom tyrosinase inhibitory activity, which was five to nine times stronger than for arbutin. Considering the ring *A* oxygenated pattern of isoflavone, **3** (the only isoflavone that was only oxygenated at C(6)) showed potent antityrosinase activity with IC_{50} values of 45.8 ± 1.8 and 52.3 ± 8.0 μM in the two test intervals. In the 7-oxygenated series, isoformononetin (**5**) also possessed notable antityrosinase activity (IC_{50} 38.1 ± 8.6 and 54.4 ± 1.9 μM), whereas the strongest inhibitor among the 7,8-dioxygenated series (*i.e.*, **1**, **6**–**8**, and **16**–**19**) was 8-hydroxydaidzein (**1**; IC_{50} 45.0 ± 0.9 and 42.3 ± 6.6 μM). Texasin (**20**) was the most prominent mushroom tyrosinase inhibitor in the 6,7-dioxygenated series, whereas prunetin (**13**), which belongs to the 5,7-dioxygenated series, showed only moderate activity (IC_{50} 113.9 ± 8.5 and 105.9 ± 11.9 μM). Among the C(7)-methoxylated series (*i.e.*, **5**, **8**, **9**, **13**, and **19**), the order of antityrosinase activities was **5** > **19** > **13** > **9** > **8**.

Based on the results shown in *Table 2*, the SAR concerning the antityrosinase activities of the tested isoflavones can be hardly defined. It can be noted that isoflavones having free OH groups at C(6), C(7), or C(4′) usually exhibited potent antityrosinase activities (*Table 2*). However, the activity becomes irregular when both the C(7) and C(4′) were hydroxylated in the same scaffold; for example, both genistein (**14**) and daidzein (**15**) were inactive, whereas 6,4′-dihydroxy-7,8-dimethylisoflavone (**3**), 8-hydroxydaidzein (**1**), and 6-hydroxydaidzein (**2**) were powerful tyrosinase inhibitors (*Table 2*). Overall, the investigated isoflavones can be divided into non-*ortho*-dioxygenated and *ortho*-dioxygenated (*i.e.*, **1**, **2**, **6**–**9**, and **16**–**22**) series to differentiate their tyrosinase inhibitory properties.

Among the non-*ortho*-dioxygenated series, **4**, **10**–**12**, and **23**–**25** were isoflavones with an OH group at C(7) that exhibited mild mushroom tyrosinase inhibitory effects. On the other hand, isoflavones **3**, **5**, and **13** were C(4′)-hydroxylated isoflavones that showed more effective inhibition of mushroom tyrosinase activities. Apparently, the tyrosinase inhibitory activities of both genistein (**14**) and daidzein (**15**) were restored by *O*-methylation either at C(7) or at C(4′). In addition, a free OH group at C(6) of an isoflavone seems to be compatible with a OH group at C(4′) in the case of **3**, which is

also a potential tyrosinase inhibitor. The OH group at C(4′) or C(7) of an isoflavone might have imitated the tyrosyl ring of the enzyme substrate when it contacts tyrosinase. Briefly, in isoflavones having a C(4′)-hydroxylated ring *B*, the enzyme-substrate binding seems to be more feasible than in C(7)-hydroxylated isoflavones in the non-*ortho*-dihydroxylated series based on the IC_{50} values (*Table 2*).

In this study, compounds of the *ortho*-dihydroxylated series (*i.e.*, **1**, **2**, **6**–**9**, and **16**–**22**) with OH groups at C(7) and C(8) or at C(6) and C(7) appeared to be good mushroom tyrosinase inhibitors, with the exception of the C(7)-methoxylated derivatives (*i.e.*, **8**, **9**, **19**, **21**, and **22**). However, *O*-methylation at C(4′) showed an inconsistent substitution effect on antityrosinase activity; for example, retusin (**17**) retained *ca.* 80% of the activity of 8-hydroxydaidzein (**1**), whereas texasin (**20**) was 60% more active than 6-hydroxydaidzein (**2**). This outcome confirmed that the mushroom tyrosinase inhibitory manner of the *ortho*-dihydroxylated series was entirely different from that of the non-*ortho*-dihydroxylated series. Basically, O-bearing substituents at C(4′) of ring *B* in the series seem to contribute to the antityrosinase activity, whereas the same substituents (OH or MeO) at C(3′) and C(2′) of ring *B* of an isoflavone tend to attenuate the tyrosinase inhibitory effect. Taken together, when an isoflavone molecule is having a lipophilic end combined with a phenolic terminal, the binding to mushroom tyrosinase is generally more feasible both in the non-*ortho*- and *ortho*-dihydroxylated series. Consequently, the existence of a lipophilic end in hydroxylated isoflavones may contribute to the binding to mushroom tyrosinase.

Impact on B16F10 Melanoma Cells and HaCaT Keratinocytes Viability. The critical factors of chemicals to be used as cosmeceuticals can be the non-hazardous property to skin cells and the lack of systemic adverse reaction when applied to human body. The cell viability assay on HaCaT human keratinocytes was used to check compounds for possible toxicity or occurring irritation when applied to human skin. Although B16F10 murine melanoma cells are tumor cells and if the tested compounds showed melanomacytotoxic activities, they would be valuable in antitumor research, the task was hereby focused on the inhibition of melanin biosynthesis by isoflavones *in vitro*. In other words, the tested compounds should not kill the melanoma cells to perform the cellular antityrosinase activity and subsequent inhibition on cellular melanogenesis. *Table 3* shows the impact on cell viability of both melanoma cells and keratinocytes by different concentrations of isoflavones **1**–**13** and **16**–**25**. Only isoflavones **6**, **12**, **16**, **17**, and **24** showed significant inhibitory effects on the cell viability of B16F10 melanoma cells at concentrations of 40 and 80 μM. All other isoflavones appeared to be non-cytotoxic to melanoma cells at the highest treatment concentration (80 μM). Although isoflavones **6**, **16**, and **17** also exhibited mild toxic effects to HaCaT keratinocyte cells ($<50\%$ inhibition of cell viability) at 40 μM, it became innocuous when the concentration was ≤ 20 μM. Although isoflavones **6**, **12**, **16**, **17**, and **24** showed proliferative inhibition on melanoma cells at a concentration of 40 μM, they were less toxic to keratinocytes, suggesting that isoflavones are almost non-toxic to normal cells, a criterion favored in cosmeceutical applications.

Inhibition of Cellular Melanin Formation and Tyrosinase Activity in B16F10 Melanocytes. The selected isoflavones **3**, isoformononetin (**5**), **9**, prunetin (**13**), retusin (**17**), and texasin (**20**) were usually potent mushroom tyrosinase inhibitors among their series except **9**, which was used for comparison with C(7)-methoxylated analogs (**5** and

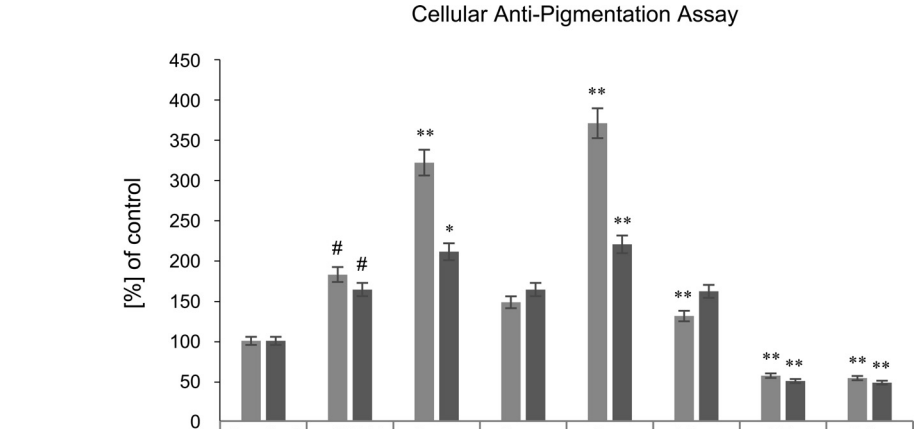
Table 3. *Cell Viability Impact of Isoflavones* **1**–**13** *and* **16**–**25** *on B16F10 Murine Melanoma Cells and HaCaT Human Keratinocytes*[a])

Compound	Cell viability [% of control]/Concentration [μM]			
	B16F10		HaCaT	
	40	80	20	40
1	101.0±2.8	98.3±4.6	97.1±4.9	94.9±5.0
2	95.1±1.0*	63.7±9.4**	97.5±3.8	95.5±7.0
3	97.6±3.9	58.1±2.9**	102.6±4.8	99.6±5.4
4	98.8±0.9	69.3±1.9**	99.1±10.7	100.7±5.8
5	92.3±6.9	61.8±0.8**	98.7±6.0	91.5±8.6
6	7.4±13.2	5.0±10.0	98.1±6.9	45.3±9.2
7	95.1±12.1	52.9±6.6	94.9±6.7	92.6±2.1*
8	86.4±9.8	62.3±11.4**	101.5±7.4	93.4±4.9
9	102.7±3.2	90.1±5.4	98.5±9.0	95.2±5.1
10	101.3±0.7	82.1±12.4	108.0±2.5**	99.8±3.6
11	99.7±6.2	100.2±3.4	95.7±6.4	94.2±6.8
12	26.7±11.1	9.9±7.5	93.3±6.1	73.9±4.7
13	97.8±2.5	90.8±9.5	97.9±3.3	88.6±6.8
16	41.6±5.8	28.3±9.5	97.7±10.4	46.3±9.1
17	35.7±1.6	3.2±14.8	99.2±5.8	40.4±7.6
18	99.2±10.2	78.2±3.3**	96.2±6.3	96.8±6.7
19	70.7±7.3**	32.5±13.8**	92.8±9.4	91.0±6.5
20	94.6±2.8*	56.6±11.8**	103.9±2.7	84.9±5.1
21	99.9±3.8	95.4±8.0	105.2±3.6	105.0±2.2**
22	99.2±3.2	100.0±5.6	99.6±3.6	98.7±6.3
23	91.5±7.7	47.3±7.7	94.1±5.2	69.9±7.6
24	86.9±7.2	7.7±12.9	98.5±7.4	73.9±3.9
25	84.6±3.0**	84.8±2.8**	98.1±5.7	97.8±4.3

[a]) Data are presented as mean ±SD ($n=4$). * $P<0.05$, ** $P<0.01$ indicates significant differences.

13) and its isomer, texasin (**20**). Because similar experiments had been performed on 8-hydroxydaidzein (**1**) [12][13], retusin (**17**) of the 7,8-dioxygenated series was chosen. The quality of cellular tyrosinase activity and melanogenesis of melanoma cells were given as percent of control (set as 100%). α-Melanocyte-stimulating hormone (α-MSH) is a melanotropin commonly used to confirm the capability of melanin biosynthesis and is a competitive target in melanoma cells. Based on the results shown in *Table 3*, the tested concentrations of selected isoflavones were the maximum non-toxic dosages to melanoma cells; this was to assure that the inhibition of cellular tyrosinase activity and the reduction of melanin deposits did not result from the cytotoxic activity of isoflavones towards the cells.

Retusin (**17**; 10 μM) and texasin (**20**; 40 μM) attenuated the cellular melanin formation from 182.6 (α-MSH) to 56.6 and 53.5%, respectively, which means *ca.* 70% reduction compared to the α-MSH group (*Fig. 1*). The pigmentation conditions of both retusin- (**17**) and texasin (**20**)-treated groups showed apparent lightening effects that could be verified with the naked eye as shown in *Fig. 2*. Isoformononetin (**5**) and prunetin (**13**) showed marginal inhibition of cellular melanin formation, but without affecting the cellular tyrosinase activity when compared with that of the α-MSH group.



	Control	α-MSH	3	5	9	13	17	20
Melanin content	100	182.6	321.9	148.1	371	131	56.6	53.5
Tyrosinase activity	100	164	210.8	164	220	161.8	49.9	47.9

Fig. 1. *Cellular anti-pigmentation effect of selected isoflavones on α-MSH-stimulated B16F10 murine melanoma cells.* Treatment: **3**, **5**, **9**, **13**, and **20** were 40 μM, and **17** was 10 μM (maximum non-toxic dosages to melanoma cells). The quality of cellular tyrosinase activity and melanin deposits influenced by isoflavones were compared with those of the α-MSH group, values are given as percentage of control (standardized as 100%). Data represents mean ±SD. # Indicates significant differences from the control group. * $P<0.05$, ** $P<0.01$ indicates significant differences from the α-MSH-treated group.

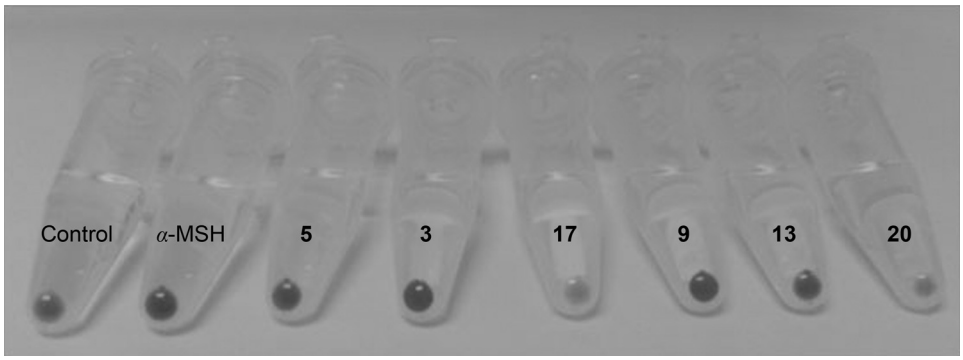


Fig. 2. *Melanin deposits of B16F10 murine melanoma cells treated by isoflavones* **3** (40 μM), *isoformononetin* (**5**, 40 μM), **9** (40 μM), *prunetin* (**13**, 40 μM), *retusin* (**17**, 10 μM), *and texasin* (**20**, 40 μM) *according to* Fig. 1. Both the melanin coloring of retusin (**17**) and texasin (**20**) appeared lighter than that of any other compound apparently.

Unexpectedly, isoflavones **3** and **9** were found to enhance cellular tyrosinase activity, causing more melanogenesis of B16F10 cells (*Fig. 1*).

In this study, both retusin (**17**) and texasin (**20**) were demonstrated to eliminate *ca.* 70% of the α-MSH stimulated cellular melanin formation and tyrosinase activity at different tested concentrations. These two isoflavones seem to have an impact on the

tyrosinase translation. Interestingly, both **3** and **9** not only significantly elevated cellular melanin formation (+76 and +103% of the α-MSH group) but also increased tyrosinase activity (+28.5 and +34.1% of the α-MSH group; *Fig. 1*). In addition, isoflavone **3** was found to have a similar structure to that of **9**, both have OH groups at C(4′) and C(6) as well as a lipophilic substituent at C(7) (Me *vs.* MeO, resp.). This outcome suggested that a free OH group at C(7) could be vital for inhibiting cellular tyrosinase activity and reduction of melanin formation. In contrast, when a lipophilic substituent was located at C(7) in a C(6)-hydroxylated isoflavone, it could become a cellular melanin inducer. The inconsistency between mushroom tyrosinase and cellular tyrosinase inhibition of **3**, **5**, **9**, and **13** confirmed that the OH group at C(7) of isoflavone could be the key functional group to perform reduction in cellular melanogenesis when compared with both **17** and **20** as well as the reported cellular melanin formation inhibitors including 8-hydroxydaidzein (**1**) [12][13], 7,3′,4′-trihydroxyisoflavone [12], and calycosin [16]. The binding of mushroom tyrosinase and phenolic substrates should have been performed randomly under non-cellular conditions. However, this binding in biosystems could have been limited by several elements such as membrane permeability or bioavailability of the substrate, other ligand-substrate binding competition, cellular xenobiotic metabolism, and cellular elimination. For example, oxidative demethylating metabolism could have transformed isoformononetin (**5**) and prunetin (**13**) into daidzein (**15**) and genistein (**14**), respectively, which were inactive mushroom tyrosinase inhibitors (*Table 2*) [10][12]. To determine whether cellular tyrosinase suppressive or expressive properties were conducted in melanoma cells, advanced tests, such as using western blotting to determine if enzyme translation was interfered by these isoflavones, would be necessary. Although having a higher IC_{50} value (51.8±4.6 μM) in mushroom tyrosinase inhibitory assay, retusin (**17**) showed almost the same inhibitory effect both on cellular tyrosinase activity and on melanogenesis of B16F10 cells at a lower treatment concentration (10 μM) when compared with texasin (**20**; 40 μM). Both the interfering of tyrosinase translation and the tyrosinase suicide-substrate effect of 7,8-dihydroxylated isoflavones might have contributed to the cellular antityrosinase efficacy of retusin (**17**) [10][13][14].

Conclusions. – 8-Hydroxydaidzein (**1**) and 6-hydroxydaidzein (**2**), both natural products isolated from fermented soy foodstuffs [10–12], are known to be potential mushroom tyrosinase inhibitors. However, their yields and natural sources are limited [10–15][18]. The present one-pot synthesis of isoflavones makes the compounds of the 6,7- and 7,8-dioxygenated series more accessible [21], especially the 7,8-dihydroxylated isoflavones that usually can be obtained in high yields. Retusin (**17**) and texasin (**20**), the C(4′)-methoxylated derivatives of **1** and **2**, might have the advantage of being absorbed by melanoma cells due to the expression of σ-receptors [25][26]. The design of σ-receptor–ligand bonded imaging agents demonstrated that 4-MeO-containing benzamide derivatives exhibited better uptake by melanoma cells than related 4-OH derivatives, suggesting that the σ-receptor–ligand binding effect could have facilitated the absorption of retusin (**17**) and texasin (**20**) [27]. In fact, bioavailability studies in animal models demonstrated that *O*-methylated polyphenolic isoflavones such as formononetin and biochanin A (**24**) showed a higher intestinal absorption rate and

lower hepatic conjugative metabolism than related polyphenols [28]. The better bioavailability might be the advantage of these two isoflavones to contribute to a more powerful inhibition on cellular melanogenesis. Although 6-hydroxydaidzein (**2**) was reported to be a powerful inhibitor of mushroom tyrosinase [10][11], it failed to reduce the cellular melanin formation in another study [12]. Texasin (**20**) showed not only powerful inhibition of mushroom tyrosinase but also was capable of reducing the cellular melanin formation, confirming that the MeO–C(4′) group (or *p*-anisyl ring *B*) had a better bioavailability than that of the OH–C(4′) group when compared with 6-hydroxydaidzein (**2**) [12]. In summary, the present findings have revealed that retusin (**17**) and texasin (**20**) are both potent free-radical scavengers and efficient inhibitors of cellular melanogenesis under non-hazardous concentrations, indicating their potential for the development of advanced cosmeceuticals.

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Experimental Part

General. The synthetic materials, including resorcinol, pyrogallol, 3-methoxycatechol, 3-methoxyphenol, 2,3-dimethylhydroquinone, various phenylacetic acids, (3-hydroxyphenyl)acetonitrile, and methyl iodide, were purchased from *Tokyo Chemical Industry* (Tokyo, Japan). $BF_3 \cdot Et_2O$, 2-methoxyhydroquinone, phloroglucinol, pyrogallol, $MeSO_2Cl$, and DMF were purchased from *Alfa Aesar* (England). Mushroom tyrosinase (EC 1.14.18.1), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT), α-MSH, cell culture materials, arbutin, and ascorbic acid were purchased from *Sigma–Aldrich Corp.* (St. Louis, MO, USA). M.p.: *Electrothermal 9100* melting point apparatus (*Electrothermal Engineering Ltd.*, Landon, UK); uncorrected. Thin layer chromatography (TLC): *Kieselgel 60* or GF_{254} plates (SiO_2; *Merck*, Germany). Column chromatography (CC): *Kieselgel 60* (60–230 mesh; *Merck*, Germany). ^{1}H- and ^{13}C-NMR spectra: *Varian Mercury 400+* spectrometer (400 and 100 MHz, resp.; at 25°; *Varian Inc.*, Palo Alto, USA); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. EI-MS: *Shimadzu LCMS-IT-TOF* mass spectrometer (*Shimadzu Corp.*, Japan); in *m*/*z*. HR-EI-MS: *Bruker APEX II* (*Bruker Instrument*, Germany); in *m*/*z*.

Chemistry. General Procedure for the One-Pot Synthesis of Isoflavones **3**–**9**. Pyrogallol (1.26 g, 10 mmol) and (3-hydroxyphenyl)acetic acid (1.52 g, 10 mmol) were heated together with $BF_3 \cdot Et_2O$ (25 ml) at 90° for 2 h under N_2 atmosphere, then the mixture was cooled to ambient temp. After slowly adding 15 ml of DMF to the stirred mixture in ice bath (**caution:** Et_2O evolution), the mixture was heated gently. When the temp. reached 50–60°, 5 equiv. of $MeSO_2Cl$–DMF (2.87 g/1.83 g) were slowly added, and then heating at 70° was continued for 2 h. After cooling, the product was poured into 150 ml of icy aq. AcONa (12 g/100 ml) buffer and left overnight. The precipitates were extracted with AcOEt (2 × 100 ml), followed by washing with brine and H_2O, the AcOEt extract was dried (Na_2SO_4), and then condensed *in vacuo*, followed by recrystallization (purified by CC with proper solvent system if necessary) in MeOH/H_2O 1:1 to obtain **7** (1.84 g, 68%) as colorless powder.

6,4′-Dihydroxy-7,8-dimethylisoflavone (**3**). Yield: 1.64 g (58%). Colorless powder (MeOH). M.p. 287–289°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.48. ^{1}H-NMR ((D$_5$)pyridine): 2.33 (*s*, Ar–Me); 2.44 (*s*, Ar–Me); 7.27 (*d*, *J* = 8.4, H–C(3′,5′)); 7.84 (*d*, *J* = 8.4, H–C(2′,6′)); 8.05 (*s*, H–C(5)); 8.24 (*s*, H–C(2)); 11.74 (br. *s*, OH); 12.12 (br. *s*, OH). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 282 (M^+) [17].

7,3′-Dihydroxyisoflavone (**4**). Yield: 1.51 g (61%). Colorless needles (MeOH). M.p. 301°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.43. ^{1}H-NMR (CDOD$_3$): 6.80 (*ddd*, *J* = 0.8, 2.4, 8.0, H–C(4′)); 6.85 (*d*, *J* = 2.0, H–C(8)); 6.93 (*dd*, *J* = 2.0, 8.8, H–C(6)); 6.96 (*dt*, *J* = 1.2, 8.0, H–C(6′)); 6.99 (*t*, *J* = 2.0,

H–C(2′)); 7.23 (*t*, *J* = 8.0, H–C(5′)); 8.05 (*d*, *J* = 8.8, H–C(5)); 8.15 (*s*, H–C(2)). ^{13}C-NMR ($CDOD_3$): see *Table 1*. EI-MS: 254 (M^+) [21].

4′-Hydroxy-7-methoxyisoflavone (= *Isoformononetin*; **5**). Yield: 1.29 g (48%). Colorless granules (MeOH). M.p. 226–228°. R_f (AcOEt/hexane/acetone 3.5 : 6 : 0.5) 0.44. ^{1}H-NMR ((D_5)pyridine): 3.76 (*s*, MeO); 6.99 (*d*, *J* = 2.4, H–C(8)); 7.05 (*dd*, *J* = 2.4, 8.8, H–C(6)); 7.29 (*d*, *J* = 8.8, H–C(3′,5′)); 7.81 (*d*, *J* = 8.8, H–C(2′,6′)); 8.20 (*s*, H–C(2)); 8.39 (*d*, *J* = 8.8, H–C(5)); 11.75 (br., OH). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 268 (M^+) [29].

7,8-Dihydroxy-3′-methoxyisoflavone (**6**). Yield: 2.04 g (72%). Colorless powder (MeOH/H_2O). M.p. 253°. R_f (AcOEt/hexane/MeOH 4 : 5.5 : 0.5) 0.41. ^{1}H-NMR ((D_5)pyridine): 3.71 (*s*, MeO); 6.99–7.03 (*m*, H–C(4′)); 7.37–7.39 (*m*, H–C(5′,6′)); 7.38 (*d*, *J* = 8.4, H–C(6)); 7.54 (*dd*, *J* = 1.2, 2.0, H–C(2′)); 8.10 (*d*, *J* = 8.4, H–C(5)); 8.36 (*s*, H–C(2)). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 284 (M^+). HR-EI-MS: 284.0688 (M^+, $C_{16}H_{12}O_5^+$; calc. 284.0685) [30].

7,8,3′-Trihydroxyisoflavone (**7**). Yield: 1.84 g (68%). Colorless powder (MeOH/H_2O). M.p. 298°. R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.40. ^{1}H-NMR ((D_5)pyridine): 7.19 (*ddd*, *J* = 2.0, 2.4, 8.0, H–C(4′)); 7.30 (*dt*, *J* = 1.2, 7.6, H–C(6′)); 7.33 (*d*, *J* = 8.8, H–C(6)); 7.37 (*t*, *J* = 8.0, H–C(5′)); 7.78 (*t*, *J* = 1.6, H–C(2′)); 8.06 (*d*, *J* = 8.8, H–C(5)); 8.33 (*s*, H–C(2)). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 270 (M^+). HR-EI-MS: 270.0533 (M^+, $C_{15}H_{10}O_5^+$; calc. 270.0528).

8,3′,4′-Trihydroxy-7-methoxyisoflavone (**8**). Yield: 1.95 g (65%). Colorless powder (MeOH/H_2O). M.p. 280° (dec.). R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.23. ^{1}H-NMR ((D_5)pyridine): 3.83 (*s*, MeO); 7.12 (*d*, *J* = 8.8, H–C(6)); 7.31 (*dd*, *J* = 2.0, 8.0, H–C(6′)); 7.34 (*d*, *J* = 8.0, H–C(5′)); 7.90 (*d*, *J* = 2.0, H–C(2′)); 8.06 (*d*, *J* = 8.8, H–C(5)); 8.37 (*s*, H–C(2)). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 300 (M^+) [17].

6,4′-Dihydroxy-7-methoxyisoflavone (**9**). Yield: 1.28 g (45%). Colorless needles (MeOH/H_2O). M.p. 325° (dec.). R_f (AcOEt/hexane/acetone 4 : 5.5 : 0.5) 0.30. ^{1}H-NMR ((D_5)pyridine): 3.87 (*s*, MeO); 7.09 (*s*, H–C(8)); 7.31 (*d*, *J* = 8.8, H–C(3′,5′)); 7.81 (*d*, *J* = 8.8, H–C(2′,6′)); 8.22 (*s*, H–C(5)); 8.23 (*s*, H–C(2)). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 284 (M^+) [17][29].

General Cyclization Procedure of 2-Hydroxydeoxybenzoins to Isoflavones. This method was carried out using the respective deoxybenzoins reported previously to obtain major isoflavones **1**, **2**, **12**, and **14**–**25** (*Scheme*) [19][20]. Biochanin A (**24**) was synthesized by dissolving 2,4,6-trihydroxy-4′-methoxydeoxybenzoin (1.37 g, 5 mmol) in DMF (6 ml), followed by slowly adding $BF_3 \cdot Et_2O$ (10 ml) in ice bath (**caution:** Et_2O evolution). After removing the ice bath, the mixture was gently heated at 50–60°, and then 4 equiv. of $MeSO_2Cl$–DMF (2.29 g/1.46 g) were slowly added, followed by heating at 70° for 2 h. The quenching process and product purification were carried out as described above for the synthesis of **7**.

7,8,4′-Trihydroxyisoflavone (= *8-Hydroxydaidzein*; **1**). Yield: 880 mg (65%). Colorless powder (MeOH/H_2O). M.p. 331–332° (dec.). R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.37. ^{1}H-NMR ($CDOD_3$): 6.84 (*d*, *J* = 8.8, H–C(3′,5′)); 6.95 (*d*, *J* = 8.8, H–C(6)); 7.36 (*d*, *J* = 8.8, H–C(2′,6′)); 7.58 (*d*, *J* = 8.8, H–C(5)); 8.18 (*s*, H–C(2)). ^{13}C-NMR ($CDOD_3$): see *Table 1*. EI-MS: 270 (M^+) [10–12][14][15][17][18].

6,7,4′-Trihydroxyisoflavone (= *6-Hydroxydaidzein*; **2**). Yield: 811 mg (60%). Colorless needles (MeOH/H_2O). M.p. 322–323° (dec.). R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.43. ^{1}H-NMR ((D_5)pyridine): 7.26 (*d*, *J* = 8.8, H–C(3′,5′)); 7.27 (*s*, H–C(8)); 7.81 (*d*, *J* = 8.8, H–C(2′,6′)); 8.16 (*s*, H–C(2)); 8.24 (*s*, H–C(5)). ^{13}C-NMR ((D_5)pyridine): see *Table 1*. EI-MS: 270 (M^+) [10–12][14][15][17][18][29].

5,7-Dihydroxy-3′-methoxyisoflavone (= *Mutabilein*; **12**). Yield: 1.23 g (87%). Colorless needles (MeOH/H_2O). M.p. 204°. R_f (AcOEt/hexane/MeOH 4 : 5.5 : 0.5) 0.69. ^{1}H-NMR ((D_6)acetone): 3.83 (*s*, MeO); 6.30 (*d*, *J* = 2.0, H–C(6)); 6.43 (*d*, *J* = 2.0, H–C(8)); 6.96 (*ddd*, *J* = 1.2, 2.4, 8.4, H–C(4′)); 7.17 (*ddd*, *J* = 0.8, 1.2, 7.6, H–C(6′)); 7.20 (*dd*, *J* = 2.4, 2.4, H–C(2′)); 7.35 (*t*, *J* = 8.0, H–C(5′)); 8.25 (*s*, H–C(2)); 9.64 (*s*, HO–C(7)); 12.98 (*s*, HO–C(5)). ^{13}C-NMR ((D_6)acetone): see *Table 1*. EI-MS: 284 (M^+) [31].

5,7,4′-Trihydroxyisoflavone (= *Genistein*; **14**). Yield: 1.13 g (84%). Yellowish powder (MeOH/H_2O). M.p. 296–298° (dec.). R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.71. ^{1}H-NMR ((D_5)pyridine): 6.67 (*d*, *J* = 2.4, H–C(6)); 6.75 (*d*, *J* = 2.4, H–C(8)); 7.28 (*d*, *J* = 8.8, H–C(3′,5′)); 7.72 (*d*, *J* = 8.8, H–C(2′,6′));

8.13 (*s*, H–C(2)); 11.85 (br., OH); 13.16 (br., OH); 13.67 (*s*, HO–C(5)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 270 (M^+) [10–12][17][18][21].

7,4′-Dihydroxyisoflavone (= *Daidzein*; **15**). Yield: 670 mg (53%). Colorless powder (MeOH/H$_2$O). M.p. 325°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.28. ^{1}H-NMR (CDOD$_3$): 6.84 (*d*, *J* = 8.8, H–C(3′,5′)); 6.85 (*d*, *J* = 2.0, H–C(8)); 6.94 (*dd*, *J* = 2.4, 8.8, H–C(6)); 7.36 (*d*, *J* = 8.8, H–C(2′,6′)); 8.05 (*d*, *J* = 8.4, H–C(5)); 8.13 (*s*, H–C(2)). ^{13}C-NMR (CDOD$_3$): see *Table 1*. EI-MS: 254 (M^+) [10–12][18][21].

7,8-Dihydroxyisoflavone (**16**). Yield: 1.38 g (93%). Colorless powder (MeOH/H$_2$O). M.p. 240–241°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.38. ^{1}H-NMR ((D$_6$)acetone): 7.03 (*d*, *J* = 8.4, H–C(6)); 7.35 (*dt*, *J* = 1.6, 7.6, H–C(4′)); 7.41 (*dt*, *J* = 1.6, 7.2, H–C(3′,5′)); 7.60 (*dm*, *J* = 8.0, H–C(2′,6′)); 7.62 (*d*, *J* = 8.4, H–C(5)); 8.82 (br., OH); 9.23 (br., OH). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 254 (M^+) [17][29].

7,8-Dihydroxy-4′-methoxyisoflavone (= *Retusin*; **17**). Yield: 1.10 g (77%). Colorless needles (MeOH/H$_2$O). M.p. 275°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.43. ^{1}H-NMR ((D$_6$)acetone): 3.83 (*s*, MeO); 6.97 (*d*, *J* = 8.8, H–C(3′,5′)); 7.51 (*d*, *J* = 8.8, H–C(5)); 7.55 (*d*, *J* = 8.8, H–C(2′,6′)); 8.19 (*s*, H–C(2)); 8.61 (*s*, OH); 9.07 (*s*, OH). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 284 (M^+) [17][21][29].

7,8-Dihydroxy-2′-methoxyisoflavone (**18**). Yield: 1.22 g (86%). Colorless powder (MeOH). M.p. 254–255°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.36. ^{1}H-NMR ((D$_6$)acetone): 3.77 (*s*, MeO); 6.98 (*dt*, *J* = 1.2. 8.4, H–C(5′)); 7.01 (*d*, *J* = 8.8, H–C(6)); 7.06 (*dd*, *J* = 1.2, 8.0, H–C(3′)); 7.30 (*dt*, *J* = 1.6, 7.6, H–C(6′)); 7.35 (*dt*, *J* = 1.6, 7.6, H–C(4′)); 7.58 (*d*, *J* = 8.4, H–C(5)); 8.07 (*s*, H–C(2)); 8.86 (br., 2 OH). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 284 (M^+). HR-EI-MS: 284.0690 (M^+, $C_{16}H_{12}O_5^+$; calc. 284.0685).

8,4′-Dihydroxy-7-methoxyisoflavone (**19**). Yield: 1.04 g (73%). Colorless needles (MeOH). M.p. 311°. R_f (AcOEt/hexane/MeOH 4.5:5:0.5) 0.38. ^{1}H-NMR ((D$_5$)pyridine): 7.14 (*d*, *J* = 8.8, H–C(6)); 7.27 (*d*, *J* = 8.4, H–C(3′,5′)); 7.80 (*d*, *J* = 8.4, H–C(2′,6′)); 8.08 (*d*, *J* = 8.8, H–C(5)); 8.34 (*s*, H–C(2)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 284 (M^+). HR-EI-MS: 284.0687 (M^+, $C_{16}H_{12}O_5^+$; calc. 284.0685).

6,7-Dihydroxy-4′-methoxyisoflavone (= *Texasin*; **20**). Yield: 782 mg (55%). Colorless needles (MeOH/H$_2$O). M.p. 258–260°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.41. ^{1}H-NMR ((D$_5$)pyridine): 3.67 (*s*, MeO); 7.05 (*d*, *J* = 8.8, H–C(3′,5′)); 7.28 (*s*, H–C(8)); 7.78 (*d*, *J* = 8.8, H–C(2′,6′)); 8.15 (*s*, H–C(5)); 8.23 (*s*, H–C(2)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 284 (M^+) [17][29].

6-Hydroxy-7-methoxyisoflavone (**21**). Yield: 870 mg (65%). Colorless needles (MeOH). M.p. 271–272°. R_f (AcOEt/hexane/acetone 3.5:6:0.5) 0.38. ^{1}H-NMR ((D$_5$)pyridine): 3.88 (*s*, MeO); 7.11 (*s*, H–C(8)); 7.37 (*td*, *J* = 7.6, 2.0, H–C(4′)); 7.46 (*t*, *J* = 8.0, H–C(3′,5′)); 7.84 (*dd*, *J* = 2.0, 8.4, H–C(2′,6′)); 8.20 (*s*, H–C(5)); 8.24 (*s*, H–C(2)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 268 (M^+) [17][29].

6-Hydroxy-7,4′-dimethoxyisoflavone (**22**). Yield: 1.12 g (75%). Yellowish needles (MeOH). M.p. 254–255°. R_f (AcOEt/hexane/acetone 3.5:6:0.5) 0.33. ^{1}H-NMR ((D$_5$)pyridine): 3.69 (*s*, MeO); 3.87 (*s*, MeO); 7.07 (*d*, *J* = 8.8, H–C(3′,5′)); 7.11 (*s*, H–C(8)); 7.81 (*d*, *J* = 8.8, H–C(2′,6′)); 8.21 (*s*, H–C(5)); 8.23 (*s*, H–C(2)); 11.97 (br., OH). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 298 (M^+) [17][29].

5,7-Dihydroxyisoflavone (**23**). Yield: 1.03 g (81%). Yellowish needles (MeOH/H$_2$O). M.p. 199–201°. R_f (AcOEt/hexane/acetone 3.5:6:0.5) 0.61. ^{1}H-NMR ((D$_6$)acetone): 6.30 (*d*, *J* = 2.4, H–C(6)); 6.43 (*d*, *J* = 2.0, H–C(8)); 7.39–7.46 (*m*, H–C(3′,4′,5′)); 7.61 (*dd*, *J* = 1.2, 8.4, H–C(2′,6′)); 8.23 (*s*, H–C(2)); 9.78 (br., HO–C(7)); 12.96 (*s*, HO–C(5)). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 254 (M^+) [17][21][29].

5,7-Dihydroxy-4′-methoxyisoflavone (= *Biochanin A*; **24**). Yield: 1.29 g (91%). Colorless powder (MeOH/H$_2$O). M.p. 218–219°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.68. ^{1}H-NMR ((D$_5$)pyridine): 3.71 (*s*, MeO); 6.67 (*d*, *J* = 2.4, H–C(6)); 6.76 (*d*, *J* = 2.4, H–C(8)); 7.09 (*d*, *J* = 8.8, H–C(3′,5′)); 7.71 (*d*, *J* = 8.8, H–C(2′,6′)); 8.14 (*s*, H–C(2)); 13.61 (*s*, HO–C(5)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 284 (M^+) [17][21].

5,7-Dihydroxy-2′-methoxyisoflavone (**25**). Yield: 1.28 g (90%). Yellow solid (CH_2Cl_2/MeOH). M.p. 201–203°. R_f (AcOEt/hexane/MeOH 4:5.5:0.5) 0.67. ^{1}H-NMR ((D$_6$)acetone): 3.79 (*s*, MeO); 6.29 (*d*, *J* = 2.4, H–C(6)); 6.43 (*d*, *J* = 2.4, H–C(8)); 7.00 (*dt*, *J* = 1.2, 7.6, H–C(5′)); 7.08 (*d*, *J* = 8.4, H–C(3′)); 7.31

(*dd*, *J* = 2.0, 7.6, H–C(6′)); 7.38 (*dt*, *J* = 1.6, 8.4, H–C(4′)); 8.06 (*s*, H–C(2)); 9.74 (*s*, OH); 12.97 (*s*, HO–C(5)). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 284 (M^+) [32].

Preparation of **11**. In brief, **12** (568 mg) was heated under reflux in HBr (48%)/AcOH 1 : 1 (30 ml) for 5 h. After cooling, the product was poured into 120 ml of icy water and then extracted with AcOEt (2 × 100 ml). The extract was combined and washed with brine and H_2O, dried ($MgSO_4$), and then recrystallized to obtain **11** after condensation.

5,7,3′-Trihydroxyisoflavone (**11**). Yield: 680 mg (84%). Yellowish needles (MeOH/H_2O). M.p. 253°. R_f (AcOEt/hexane/MeOH 4.5 : 5 : 0.5) 0.73. ^{1}H-NMR ((D$_6$)acetone): 6.29 (*d*, *J* = 2.4, H–C(6)); 6.42 (*d*, *J* = 2.4, H–C(8)); 6.87 (*ddd*, *J* = 1.2, 2.4, 8.4, H–C(4′)); 7.04 (*ddd*, *J* = 0.8, 1.6, 7.6, H–C(6′)); 7.12 (*dd*, *J* = 2.0, 2.0, H–C(2′)); 7.25 (*t*, *J* = 8.0, H–C(5′)); 8.20 (*s*, H–C(2)); 10.30 (*s*, OH); 12.99 (*s*, HO–C(5)). ^{13}C-NMR ((D$_6$)acetone): see *Table 1*. EI-MS: 270 (M^+). HR-EI-MS: 270.0530 (M^+, $C_{15}H_{10}O_5^+$; calc. 270.0528) [32].

Preparation of Prunetin (**13**). In brief, genistein (**14**; 540 mg, 2 mmol) and MeI (284 mg) were dissolved in acetone (20 ml), and then stirred in the presence of K_2CO_3 (138 mg) for 2 h at ambient temp. The reaction was terminated, and the mixture was concentrated *in vacuo* to remove acetone, 25 ml of 1N HCl were added, and the resulting mixture was extracted with AcOEt (2 × 30 ml), followed by washing with H_2O and drying. The crude product was subjected to CC (SiO_2) and **13** was obtained by recrystallization.

5,4′-Dihydroxy-7-methoxyisoflavone (= *Prunetin*; **13**). Yield: 256 mg (45%). Colorless needles (MeOH). M.p. 245°. R_f (AcOEt/hexane/MeOH 4 : 5.5 : 0.5) 0.64. ^{1}H-NMR ((D$_5$)pyridine): 3.73 (*s*, MeO); 6.55 (*d*, *J* = 2.0, H–C(6)); 6.61 (*d*, *J* = 2.0, H–C(8)); 7.28 (*d*, *J* = 8.8, H–C(3′,5′)); 7.71 (*d*, *J* = 8.8, H–C(2′,6′)); 8.18 (*s*, H–C(2)); 11.86 (br., HO–C(4′)); 13.55 (*s*, HO–C(5)). ^{13}C-NMR ((D$_5$)pyridine): see *Table 1*. EI-MS: 284 (M^+) [29].

Bioassays. ABTS$^{\bullet+}$ *Radical Scavenging Assay.* The ABTS$^{\bullet+}$ scavenging assays of isoflavones were conducted according to the methods reported previously [19][33][34]. Briefly, ABTS was prepared with deionized H_2O in a concentration of 7 mM, it was then mixed with 2.45 mM $K_2S_2O_8$. A mixture containing 180 µl of ABTS and 40 µl of isoflavone or the negative control (phosphate buffered saline (PBS)), or the positive control (vitamin C) was well stirred, and then incubated for 7 min and 24 min at r.t. The absorbance of the mixture was measured at 734 nm. The 50% radical-scavenging concentration is expressed as SC_{50}.

DPPH$^{\bullet}$ *Radical Scavenging Assay.* In brief, 1 ml (0.1 mM) of DPPH$^{\bullet}$ soln. was mixed with 3 ml of isoflavone solns. of various concentrations or vitamin C (positive control) and dissolved in MeOH. The mixture was then stirred vigorously and kept at 40° for 30 min in the dark. For the baseline control, 3 ml of MeOH were used. The absorbance was measured at 517 nm. The 50% radical scavenging concentration is expressed in SC_{50} [19][33][34].

Mushroom Tyrosinase Inhibition Test. Antityrosinase activities were measured according to the method reported previously [19][33][34]. In brief, isoflavones or arbutin (positive control) were dissolved in DMSO/MeOH before dilution to various concentrations by potassium phosphate buffer (pH 6.8). Twenty µl of isoflavone solns. of various concentrations or arbutin soln. were mixed with 20 µl of mushroom tyrosinase (1000 U ml^{-1}) and 80 µl of L-tyrosine (2.0 mM) in a 96-well plate, then the mixture was diluted with buffer to a volume of 200 µl. After 30 and 60 min of incubation, the absorbance was measured at 490 nm.

Impact on B16F10 Melanoma Cells and HaCaT Keratinocytes Viability. B16F10 melanocyte (*BCRC 60031*, Taiwan) or HaCaT keratinocyte cells were cultured in *Dulbecco*'s modified *Eagle*'s medium (DMEM; *HyClone*®, USA) with 10% fetal bovine serum (*Sigma*), 2 mM L-glutamine, 200 U ml^{-1} penicillin G, 200 µg ml^{-1} streptomycin, and 0.5 µg ml^{-1} amphotericin B (*Kibbutz Beit Haemek*, Israel) under a humidified atmosphere containing 5% CO_2 at 37°. Cells were placed in 96-well plates ($1 \cdot 10^4$ cells/well). After 24 h of incubation, test samples were added and the cultures were further incubated for 48 h. Tested concentrations of isoflavones were 10, 20, 40, and 80 µM for B16F10 murine melanoma cells, and 5, 10, 20, and 40 µM for HaCaT human keratinocyte. Cell viability was determined using the XTT assay. Formazan formed from XTT in cell cultures during incubation was measured at 490 nm using a multi-well scanning spectrophotometer [34–36].

Cellular Melanin Formation and Tyrosinase Activity. The measurement of cellular melanin formation and tyrosinase activity was conducted according to the methods described by *Chen et al.* [37] and *Lan et al.* [34]. In brief, B16F10 cells ($5 \cdot 10^4$ cells/well) together with 50 nM α-MSH were placed in 24-well plates and then incubated for 24 h. After addition of isoflavones, samples were further incubated for 48 h, followed by washing twice with PBS. Melanocytes were dissolved in 100 µl of 2N NaOH and digested at 80° for 1 h to solubilize the generated melanin. Absorbance was measured at 405 nm, which was used to determine the melanin content [34][37].

For cellular tyrosinase activity inhibitory assay, B16F10 cells were treated with isoflavones for 48 h as described above. The harvested cells were washed twice with PBS, and then centrifuged at 12,000 rpm for 10 min, followed by addition of 1% *Triton X-100* to lyse the cell membrane before the mixture was subjected to further centrifugation. The supernatants were mixed with 100 µl of L-dopa (1 mg ml^{-1}) at 37° for 3 h. The absorbance was measured at 490 nm with a multi-well spectrophotometer. All experiments were performed for at least three times ($n \geq 3$), and the inhibition percentage of each compound was compared with that of control [34][37].

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