Melanogenesis Stimulation in Murine B16 Melanoma Cells by Umberiferae Plant Extracts and Their Coumarin Constituents

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Melanogenesis stimulation activities of seven ethanolic extracts obtained from Umbelliferae plants used as Chinese crude drugs, namely the roots of *Angelica dahurica* BENTH. *et* HOOK., *A. biserrata* SHEN *et* YUAN, *Notopterygium incisum* TING, *Heracleum lanatum* MICHX., and *H. candicans* WALL., and the fruits of *Cinidium monnieri* (L.) CUSSON and *C. formosanum* YABE, were examined by using cultured murine B16 melanoma cells. Among them, the extract (5, 25 μg/ml) of *H. lanatum* showed a potent stimulatory effect on melanogenesis with significant enhancement of cell proliferation in a dose-dependent manner. The melanogenesis stimulatory effects of sixteen coumarins (1—16) isolated from the seven Umbelliferae crude drugs were also examined. Among them, linear-furocoumarins [psoralen (1), xanthotoxin (2), bergapten (3), and isopimpinellin (4)] and angular-furocoumarin [sphondin (13)] exhibited potent melanogenesis stimulation activity. From the view point of structure–activity relationships, it may be assumed that a linear-furocoumarin ring having a hydrogen and/or methoxyl group at 5 and 8 positions such as 1, 2, 3 and 4 was preferable for the melanogenesis stimulation activity. The introduction of a prenyl group into the furocoumarin ring was disadvantageous. Coumarin derivatives having a simple coumarin ring were inactive.

Key words Umbelliferae plant; *Heracleum lanatum*; coumarin; murine B16 melanoma cell; stimulatory effect on melanogenesis

Gray hair is caused by a genetic predisposition, aging, decrement of melanocytes by environmental stress, and decrement of the biosynthesis of melanin pigment or melanogenesis.[1,2] Hair dye agents are used for the treatment of gray hair. However, there remain some problems with these agents, such as side effects due to the dyes. Thus, there is a need for anti-gray hair agents that exhibit satisfactory melanogenesis activity and gray hair prevention. For the development of gray hair prevention agents, we have carried out a screening program to find a potential stimulant of melanogenesis from natural resources by using cultured murine B16 melanoma cells with theophylline as a reference drug. In a preceding paper,[3] we reported that a methanolic extract from the leaves of *Piper nigrum* L. showed a significant stimulatory effect on melanogenesis in cultured murine B16 melanoma cells and that two lignans, (−)-cubebin and (−)-3,4-dimethoxy-3,4-desmethylenedioxycubebin, were isolated as its active constituents. As the next step in our herbal screening, we report here the melanogenesis stimulation effect of seven ethanolic extracts obtained from Umbelliferae plants that are used as Chinese crude drugs, namely the roots of *Angelica dahurica* BENTH. *et* HOOK. (Byakushi), *A. biserrata* SHEN *et* YUAN (To-dokkatsu), *Notopterygium incisum* TING (Kyokatsu), *Heracleum lanatum* MICHX. (Gyubi-dokkatsu), and *H. candicans* WALL. (Hakuryo-dokkatsu), and the fruits of *Cinidium monnieri* (L.) CUSSON (Jashoshi) and *C. formosanum* YABE (Taiwan-jashoshi). The melanogenesis stimulation effects of sixteen coumarins (**1**—**16**, Fig. 1) isolated from the Umberiferae crude drugs were also evaluated.

MATERIALS AND METHODS

Materials The roots of *Angelica biserrata* (To-dokkatsu), *Notopterygium incisum* (Kyokatsu), the fruits of *Cinidium monnieri* (Jashoshi) and *Cinidium formosanum* (Taiwan-jashoshi) were obtained from Osaka market. The roots of *Angelica dahurica* (Byakushi) and *Heracleum candicans* (Hakuryo-dokkatsu) were obtained from Chinese market (Yunnan Province). The roots of *Heracleum lanatum* (Gyubi-dokkatsu) were grown at the Osaka University of Pharmaceutical Sciences.

Preparation of Ethanolic Extracts Seven crude drugs (each 10 g) were extracted with EtOH (100 ml) in a water bath at 70 °C for 2 h and then filtrated. Each solution was concentrated under reduced pressure to give the corresponding extract: To-dokkatsu (1.00 g), Kyokatsu (1.45 g), Byakushi (0.70 g), Hakuryo-dokkatsu (1.00 g), Gyubi-dokkatsu (1.17 g), Jashoshi (0.40 g), and Taiwan-jashoshi (0.40 g).

Drugs Fourteen coumarins (**1**—**7**, **9**, **11**—**16**, Fig. 1) were isolated from the Umbelliferae crude drugs.[4—10] The isolation yields of each compound are depicted in Table 1. Synthetic melanin and 0.4 M HEPES buffer (pH 6.8) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Dulbecco's modified Eagle medium (D-MEM), Antibiotic–Antimycotic (a mixture of 10000 U/ml penicillin, 10000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericine B), Dulbecco's phosphate buffered saline (Ca^{2+}- and Mg^{2+}-free, CMF-D-PBS), and trypsin were purchased from Invitrogen Corp. (CA, U.S.A.). Fetal bovine serum (FBS) was purchased from ICN Biomedicals Co. (CA, U.S.A.). Theophylline, dimethyl sulfoxide (DMSO), and other organic solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TetraColor ONE assay kit was purchased from Seikagaku Co. (Tokyo, Japan).

Isolation of Heraclenin (8) and Heraclenol (10) from Hakuryo-dokkatsu (*H. candicans* WALL.) Powdered Hakuryo-dokkatsu (500 g) was successively extracted with hexane (5 l) and EtOAc (5 l) under reflux for 2 h. Each extract was evaporated under reduced pressure to give a hexane ex-

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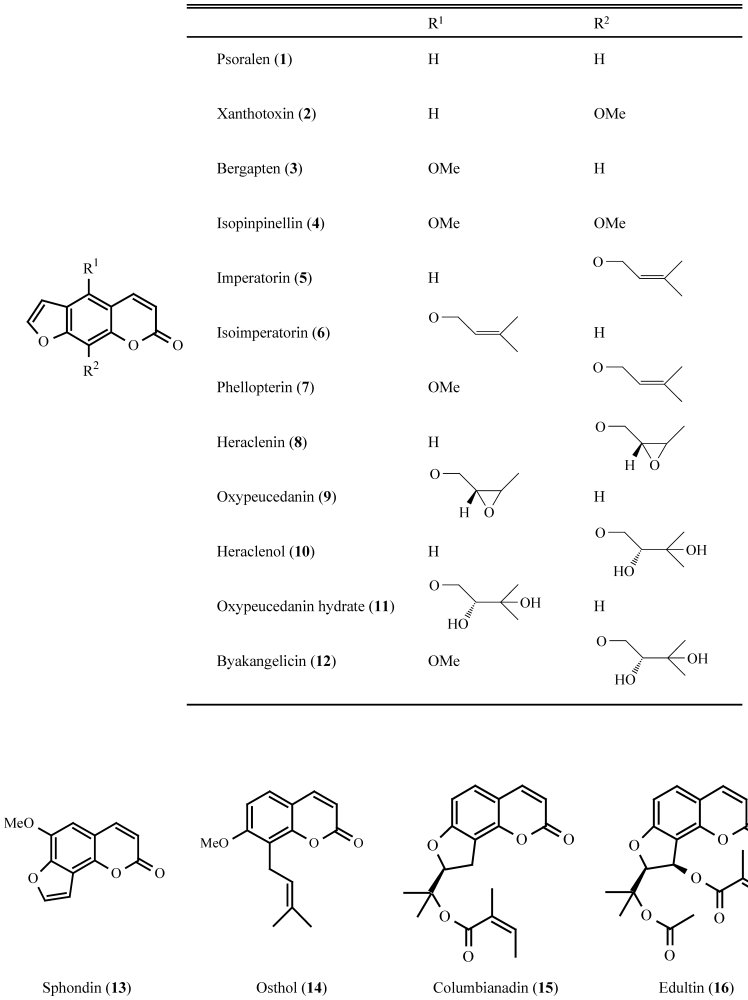

Fig. 1 . Chemical Structures of Coumarins **1**—**16**

Table 1. Isolation Yield (%) of Coumarins from Seven Umbelliferae Crude Drugs

	Byakushi (*Angelica dahurica*)[4,5]	Kyokatsu (*Notopterygium incisum*)[6]	Jashoshi (*Cnidium monnieri*)[7]	Taiwan-jashoshi (*C. formosanum*)[8]	Gyubi-dokkatsu (*Heracleum lanatum*)[9]	To-dokkatsu (*A. biserrata*)[10]	Hakuryo-dokkatsu (*H. candicans*)
Psoralen (**1**)	0.017					0.017	
Xanthotoxin (**2**)	0.018		0.0053		0.02	0.018	
Bergapten (**3**)		0.0008	0.0036	0.097	0.10	0.02	
Isopinpinellin (**4**)	0.002		0.0048	0.026	0.13		
Imperatorin (**5**)	0.0005		0.068				
Isoimperatorin (**6**)		0.11				0.0005	
Phellopterin (**7**)	0.0025						
Heraclenin (**8**)							1.16
Oxypeucedanin (**9**)	0.0025						
Heraclenol (**10**)							0.63
Oxypeucedanin hydrate (**11**)	0.01						
Byakangelicin (**12**)	0.24						
Sphondin (**13**)					0.13		
Osthol (**14**)			0.11			0.13	
Columbianadin (**15**)				0.20		0.20	
Edultin (**16**)				0.073			

tract (5.8 g) and an EtOAc extract (28.8 g). The EtOAc extract (28.7 g) was submitted to column chromatography over silica gel (900 g, 6×65 cm). Elution with hexane and EtOAc in increasing proportions (5 : 1 to 1 : 5 v/v) and the monitoring of each fraction (200 ml) with TLC gave two main furocoumarin fractions. Purification of each fraction by recrystallization from EtOAc–hexane afforded **8** (mp 107—109 °C; yield 5.8 g) and **10** (mp 116—118 °C; yield 3.15 g), respec-

tively. On the basis of comparison of the physicochemical data (mp, IR, EI-MS, ^{1}H-, ^{13}C-NMR) of **8** and **10** with those of the reported data,[11,12] **8** and **10** were identified as heraclenin and heraclenol, respectively.

Cell Culture Cultured murine B16 melanoma cell lines (B16F1) were purchased on Oct., 2002 from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan), and maintained in culture in D-MEM supplemented with 10% FBS and 1% Antibiotic–Antimycotic at 37 °C in a humidified incubator in 5% CO_2–95% air (CO_2 incubator).

Measurement of Produced Melanin in Cultured B16 Melanoma Cells The amount of melanin in cultured B16 murine melanoma cells was measured according to the method of Hill *et al.*[13] as described in one of our previous papers.[3] Briefly, trypsinized cells (2×10^4 cells/well in 1.9 ml of D-MEM) were inoculated with a pipette into 6-well plates (FALCON 353046, Becton Dickinson Labware, NJ, U.S.A.), and incubated for 24 h at 37 °C in the CO_2 incubator. After 24 h incubation, 100 μl of each sample solution was added to each well in triplicate, and the 6-well plate was incubated for 4 d at 37 °C in the CO_2 incubator. The test samples and theophylline were dissolved in DMSO/CMF-D-PBS (1 : 1, v/v), and then diluted with D-MEM to an appropriate concentration. The final concentration of DMSO was 0.1% or 0.5%. In the control group, DMSO/CMF-D-PBS (1 : 1, v/v) solution diluted with D-MEM to 0.1% or 0.5% of the final DMSO concentration was used instead of the sample solution. After incubation, the cultured medium was removed by pipette and assayed for extracellular melanin as described below. The remaining melanoma cells were trypsinized (0.25% trypsin and 0.1% EDTA at 37 °C for 5 to 10 min) and washed with 100 μl of CMF-D-PBS. The cells were then digested by adding 400 μl of 1 N NaOH and letting them stand for 16 h at room temperature. The OD at 475 nm of the resulting solution was measured using a Shimadzu UV-2400PC, and the amount of intracellular melanin was calculated by referring to a calibration curve obtained with synthetic melanin in 1 N NaOH. The cultured medium was centrifuged ($700\times\boldsymbol{g}$, 10 min at 4 °C) to give a supernatant. One milliliter of a mixture of 0.4 M HEPES buffer (pH 6.8) and EtOH (9 : 1, v/v) was added to 1 ml of the supernatant. The OD at 475 nm of the resulting solution was measured, and the amount of extracellular melanin was calculated by referring to a calibration curve obtained with synthetic melanin in a mixture of 0.4 M HEPES buffer (pH 6.8) and EtOH (9 : 1, v/v).

Assay for Cell Proliferation Cultured murine B16 melanoma cells (passage number 6) were trypsinized (0.25% trypsin and 0.1% EDTA at 37 °C for 5 to 10 min). Cells (1.5×10^3 cells/well in 80 μl of D-MEM) were inoculated with a pipette into a 96-well plate (FALCON 353072, Becton Dickinson Labware, NJ, U.S.A.), and incubated for 24 h at 37 °C in the CO_2 incubator. After 24 h incubation, 20 μl of each sample solution was added to each well in quintuplicate, and the 96-well plate was incubated for 4 d at 37 °C in the CO_2 incubator. The sample and control solutions were prepared as described above. After incubation, cell viability was assayed by a TetraColor ONE. Ten microliters of TetraColor ONE was added to each well. After 4 h, the OD at 490 nm of each well was measured using a microplate reader (Polar Galaxy, BMG Lab Technologies, Offenburg, Germany). The percentage of living cells in each well was calculated with respect to the OD value of living cells of the control group (100%).

Statistical Analysis Data points represent the mean values and standard errors ($n=3$ or 5). The experimental data were tested for statistical significance using the Bonferroni/Dunn multiple range test method.

RESULTS

Melanogenesis activity in the cultured murine B16 melanoma cells is closely related to the amount of produced melanin, which is estimated from the sum of the amount of melanin retained in the cells (intracellular melanin) and that excreted into the cultured medium (extracellular melanin). Thus, we evaluated melanogenesis activity on the basis of the sum of the amount of intracellular and extracellular melanin. According to the method of Hill *et al.*,[13] the amounts of intracellular and extracellular melanin were determined separately. The sum of the amount of melanin was calculated by the addition of both average melanin amounts, and was represented without statistical standard errors. Cell proliferation of the treated group was compared with that of the control group.

Theophylline (10 μg/ml), a reference drug, enhanced pig-

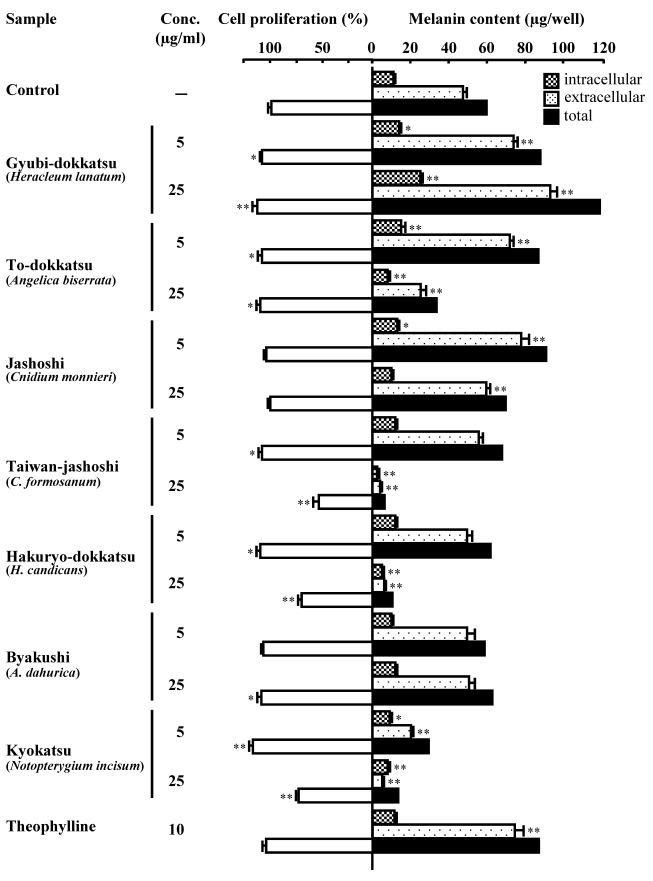

Fig. 2. Effects of Ethanol Extract, Seven Umbelliferae Crude Drugs and Theophylline on Melanin Content in Cultured B16 Murine Melanoma Cells

B16 murine melanoma cells (passage number 6) were cultured in D-MEM (final DMSO conc.; 0.1%) for 4 d, and the intracellular and extracellular amounts of melanin were assayed. Total melanin is the sum of intracellular and extracellular melanin amounts. The bars in the melanin content columns indicate the mean±S.E. of 3 wells. $*p<0.05$ and $**p<0.01$: statistically significant *vs.* the control group. The bars in the cell proliferation indicate the mean±S.E. of 5 wells. $*p<0.05$ and $**p<0.01$: statistically significant *vs.* the control group.

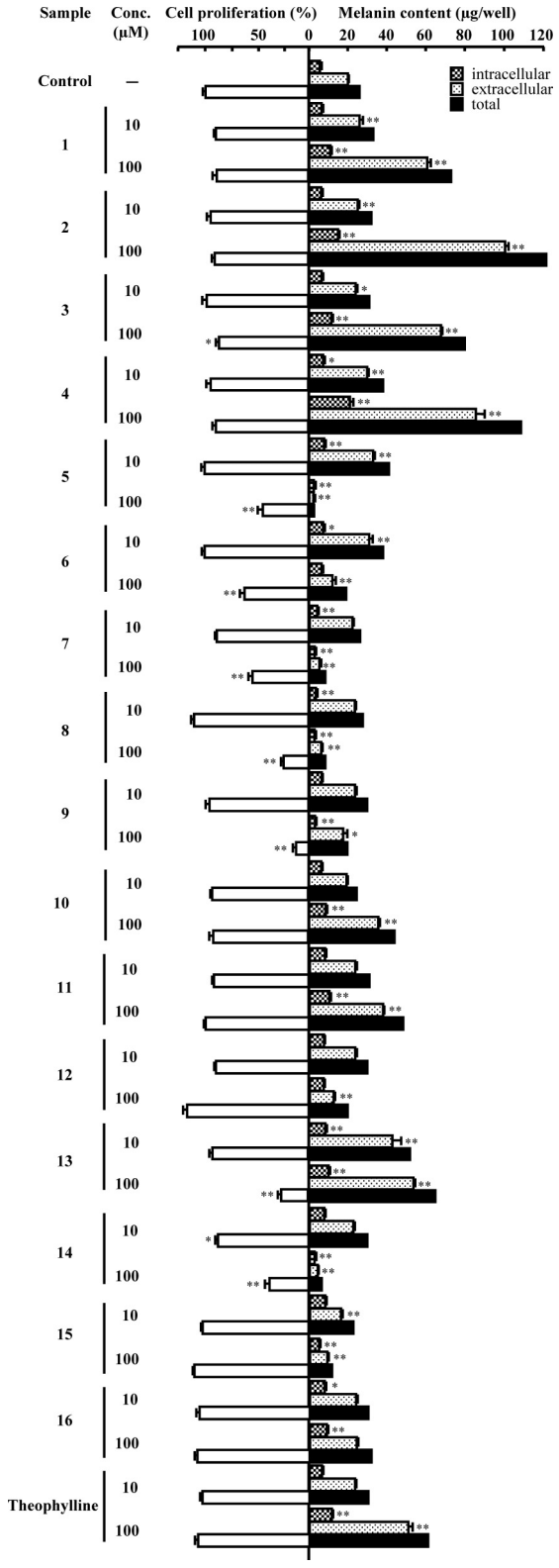

Fig. 3. Effects of Coumarins **1**—**16** from Seven Umbelliferae Crude Drugs and Theophylline on Melanin Content in Cultured B16 Murine Melanoma Cells

B16 murine melanoma cells (passage number 6) were cultured in D-MEM (final DMSO conc.; 0.5%) for 4 d and the intracellular and extracellular amounts of melanin were assayed. Total melanin is the sum of intracellular and extracellular melanin amounts. The bars in the melanin content columns indicate the mean±S.E. of 3 wells. $*p<0.05$ and $**p<0.01$: statistically significant *vs.* the control group. The bars in the cell proliferation indicate the mean±S.E. of 5 wells. $*p<0.05$ and $**p<0.01$: statistically significant *vs.* the control group.

mentation, but the drug did not show any significant effects on cell proliferation. The stimulatory effect of seven ethanolic extracts (5, 25 μg/ml) from the seven Umbelliferae plants on melanogenesis in murine B16 melanoma cells and their effects on cell proliferation are shown in Fig. 2. The extracts from Gyubi-dokkatsu, To-dokkatsu, and Jashoshi exhibited significant activity at a lower concentration (5 μg/ml). The extracts from To-dokkatsu and Jashoshi showed a tendency to suppress melanin production at a higher concentration (25 μg/ml). The extracts from Taiwan-jashoshi and Hakuryo-dokkatsu had a significant inhibitory effect on both melanin production and cell proliferation at the higher concentration of 25 μg/ml. The extracts from Hakuryo-dokkatsu and Byakushi had no effect on melanin production at the lower concentration of 5 μg/ml. Kyokatsu extract even suppressed melanin production at the lower concentration of 5 μg/ml.

The melanogenesis activity of these coumarins (**1**—**16**) was evaluated at concentrations of 10 and 100 μM. The results are shown in Fig. 3. Psoralen (**1**), xanthotoxin (**2**), isopimpinellin (**4**), heraclenol (**10**), oxypeucedanin hydrate (**11**), and edultin (**16**) significantly increased melanin production without any effects on cell proliferation. Compounds, **2** and **4** showed the most potent activity. Bergapten (**3**), imperatorin (**5**), isoimperatorin (**6**), and sphondin (**13**) exhibited significant activity at the lower concentration (10 μM), while they suppressed cell proliferation at the higher concentration (100 μM). Phellopterin (**7**), heraclenin (**8**), and oxypeucedanin (**9**) suppressed both melanin production and cell proliferation at the higher concentration of 100 μM. Byakangelicin (**12**) showed an inhibitory effect on melanogenesis at 100 μM without suppression of cell proliferation. Osthol (**14**) inhibited cell proliferation even at the lower concentration of 10 μM. Columbianadin (**15**) decreased melanin production without any effects on cell proliferation.

DISCUSSION

The reference drug, theophylline, enhances pigmentation. It has been reported that theophylline increases both tyrosinase- and gamma-glutamyl transpeptidase (GGT)-reactive cells, resulting in enhanced pigmentation in cultured murine B16 melanoma cells.[14] Many furocoumarins have been reported to be potent photosensitizing agents. Xanthoxin (**2**) and bergapten (**3**) are used as a stimulus of melanin deposition in several suntan preparations for the treatment of vitiligo.[15] The inhibitory effect of esclin, a coumarin glycoside, on melanogenesis in B16 melanoma cells at a concentration of 10 μM has been reported.[16] The melanogenesis stimulatory effect of other furocoumarins in B16 melanoma cells is not known.

The results concerning the melanogenesis activities of sixteen coumarins (**1**—**16**) are shown in Fig. 3. Four linear-furocoumarins (**1**—**4**) and angular-furocoumarin (**13**) exhibited potent stimulatory activity. Two linear-prenylfurocoumarins (**5**, **6**) exhibited weak stimulatory effects. Two oxygenated linear-prenylfurocoumarins (**10**, **11**) had weak stimulatory effects without any effects on cell proliferation. Another linear-prenylfurocoumarin (**7**) and two oxygenated linear-prenylfurocoumarins (**8**, **9**) suppressed both melanin production and cell proliferation at the higher concentration. Simple coumarin (**14**) and acylated angular-dihydrofurocoumarin

(**15**) demonstrated an inhibitory effect on melanogenesis. However, diacylated angular-dihydrofurocoumarin (**16**) had a weak stimulatory effect. The inhibition of cell proliferation generally accompanied with inhibition of melanogenesis, as in the cases of **5**—**9**, but angular-furocoumarin (**13**) showed stimulatory activity with inhibition of cell proliferation at the higher concentration (100 μM). Although the details of the mechanism of stimulation and/or inhibition of melanogenesis by these furocoumarins in B16 melanoma cells has not been fully elucidated, the effects of furocoumarins on tyrosinase and its related enzymes are under examination.

From the view point of the structure–activity relationship, it may be assumed that a linear-furocoumarin ring having a hydrogen and/or methoxyl group at the 5 and 8 positions, such as psoralen (**1**), xantoxin (**2**), bergapten (**3**), and isopinpinellin (**4**), is preferable for stimulatory activity. The introduction of prenyl or oxygenated prenyl groups into the linear-furocoumarin ring was not advantageous. Coumarin derivatives having a simple coumarin ring were insufficient for the stimulatory activity.

Although the tyrosinase-inhibitory activity of furocoumarin from Byakushi extract was reported,[17] the stimulation of melanogenesis by Umbelliferae plant extract has not been examined hitherto.

The significant inhibitory effect of extract obtained from Taiwan-jashoshi on melanin production at the concentration of 25 μg/ml may be attributable to its major constituent, columbianadin (**15**). Similarly, the significant inhibitory effect of the extract from Hakuryo-dokkatsu on both melanin production and cell proliferation at the concentration of 25 μg/ml may be attributable to its major constituent, heraclenin (**8**). The effects on melanogenesis and cell proliferation of Byakushi and Kyokatsu extracts cannot be fully explained by their constituents in Table 1. Since, their activities were not desirable for our purpose, further studies were suspended. The decreased stimulatory effect on melanogenesis of the extracts from To-dokkatsu at the concentration of 25 μg/ml may be attributable to its major constituents, osthol (**14**) and columbianadin (**15**).

The extracts from Gyubi-dokkatsu and Jashoshi showed significant stimulatory effects without inhibition of cell proliferation at concentrations of 5 and 25 μg/ml. The Gyubi-dokkatsu extract exhibited the most potent stimulatory activity on melanogenesis as well as on cell proliferation in a dose-dependent manner. The Jashoshi extract showed a slightly inferior stimulatory effect on melanogenesis at the concentration of 25 μg/ml.

Because four furocoumarins (**2**—**4**, **13**) are major constituents of Gyubi-dokkatsu as shown in Table 1, the melanogenesis stimulatory activity of the roots may be attributable to these furocoumarins. Thus, Gyubi-dokkatsu extract may be considered to be the most desirable extract for our target. In conclusion, it is believed that Gyubi-dokkatsu and its active furocoumarins may be useful ingredients in cosmetic preparations for the prevention of gray hair.

REFERENCES

1) Tobin D. J., Paus R., *Exp. Gerontol.*, **36**, 29—54 (2001).
2) Horikawa T., *J. Invest. Dermatol.*, **106**, 28—35 (1996).
3) Matsuda H., Kawaguchi Y., Yamazaki M., Hirata N., Naruto S., Asanuma Y., Kaihatsu T., Kubo M., *Biol. Pharm. Bull.*, **27**, 1611—1616 (2004).
4) Hata K., Kozawa M., Yen K. Y., *Yakugaku Zasshi*, **83**, 606—610 (1963).
5) Kozawa M., Baba K., Okuda K., Fukumoto T., Hata K., *Shoyakugaku Zasshi*, **35**, 90—95 (1981).
6) Kozawa M., Fukumoto M., Matsuyama Y., Baba K., *Chem. Pharm. Bull.*, **31**, 2712—2717 (1983).
7) Baba K., Hamasaki F., Tabata Y., Kozawa M., Honda G., Tabata M., *Shoyakugaku Zasshi*, **39**, 282—290 (1985).
8) Hata K., Kozawa M., Baba K., *Yakugaku Zasshi*, **92**, 1289—1294 (1972).
9) Hata K., Sano K., *Shoyakugaku Zasshi*, **18**, 26—27 (1964).
10) Kozawa M., Baba K., Matsuyama Y., *Shoyakugaku Zasshi*, **36**, 202—210 (1982).
11) Razdan T. K., Kachroo V., Harkar S., Koul G. L., *Phytochemistry*, **21**, 923—927 (1982).
12) Harkar S., Razdan T. K., Waight E. S., *Phytochemistry*, **23**, 419—426 (1984).
13) Hill S. E., Buffey J., Thody A. J., Oliver I., Bleehen S. S., Mac N. S., *Pigment Cell Res.*, **2**, 161—166 (1989).
14) Hu F., *J. Invest. Dermatol.*, **79**, 57—61 (1982).
15) Ashwood-Smith M. J., Poulton G. A., Barker M., Mildenberger M., *Nature* (London), **285**, 407—409 (1980).
16) Katagiri T., Yokoyama K., Koiso I., Matsukami M., Nakano H., *Jpn. J. Dermatol.*, **104**, 1367—1372 (1994).
17) Piao X. L., Baek S. H., Park M. K., Park J. H., *Biol. Pharm. Bull.*, **27**, 1144—1146 (2004).