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In Vitro and *in Vivo* Melanogenesis Inhibition by Biochanin A from *Trifolium pratense*

Victor C. LIN,[1,2,*] Hsiou-Yu DING,[3,*] Pin-Chin TSAI,[4] Jiumn-Yih WU,[2] Yen-Hsing LU,[5] and Te-Sheng CHANG[5,†]

[1]*Department of Urology, E-Da Hospital, 1, E-Da Rd., Jiau-Shu Village, Yan-chau District, Kaohsiung, Taiwan*
[2]*The PhD Program in Biotechnology, Institute of Biotechnology and Chemical Engineering, I-Shou University, 1, Sec. 1 Syuecheng Rd., Dashu District, Kaohsiung, Taiwan*
[3]*Institute of Cosmetics Science, Chia Nan University of Pharmacy and Science, 60, Sec. 1 Erh-Jen Rd., Jen-Te District, Tainan, Taiwan*
[4]*Department of Pharmacy, E-Da Hospital, 1, E-Da Rd., Jiau-Shu Village, Yan-chau District, Kaohsiung, Taiwan*
[5]*Department of Biological Science and Technology, National University of Tainan, 33 Sec. 2 Su-Lin St., Tainan, Taiwan*

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Our previous study showed that a methanol extract from *Trifolium pratense* exerted potent inhibitory activity on melanogenesis in mouse B16 melanoma cells. In the present study, the active compound in this Chinese herb extract was isolated and identified as biochanin A by mass spectrum, ^{1}H-NMR, and ^{13}C-NMR analysis. The inhibitory effects of biochanin A on melanogenesis were investigated *in vitro* in cultured melanoma cells and *in vivo* in zebrafish and mice. Biochanin A dose-dependently inhibited both melanogenesis and cellular tyrosinase activity in B16 cells and in zebrafish embryos. Application of a cream containing 2% biochanin A twice daily to the skin of mice also increased the skin-whitening index value after 1 week of treatment, and the increase continued for another 2 weeks. Biochanin A was confirmed as a good candidate for use as a skin-whitening agent in the treatment of skin hyperpigmentation disorders.

Key words: biochanin A; inhibition; melanogenesis; *Trifolium pretense*; tyrosinase

The skin color of animals and humans is determined mainly by the content of melanin pigment in the skin. Melanin is produced in dermal melanocytes by a process termed melanogenesis.[1)] Melanogenesis is initiated in melanosomes, the special organelles of melanocytes, with a first step of L-tyrosine oxidation to L-DOPA (L-3,4-dihydroxyphenylalanine) and then to dopaquinone, which is catalyzed by tyrosinase. This is a rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at physiological pH. Although melanin mainly plays a photoprotective role, the accumulation of abnormal amounts of melanin in different parts of the skin, which results in pigmented patches of skin, can become an esthetic problem. Hence several studies have focused on inhibition of melanogenesis and the prevention of abnormal pigmentation for cosmetic benefits.[2–4)]

In a previous study, we screened hundreds of crude extracts of Chinese herbs to identify their inhibitory activity on melanogenesis in mouse B16 melanoma cells. Among these, a methanol extract of *Trifolium pratense* showed strong inhibitory activity, with an IC_{50} value of 73.9 μg/mL, against the melanogenesis of B16 cells. In the present study, we isolated the active compound from this Chinese herb and identified the compound as biochanin A (Fig. 1) by mass spectra, ^{1}H-NMR, and ^{13}C-NMR analysis. This compound was previously isolated from several plants, and has been identified as a chemopreventive agent,[5)] a protein kinase C inhibitor,[6)] an antidiabetic agent,[7)] an anti-platelet agent,[8)] and an antiprotozoal compound.[9)] Although biochanin A has many bioactivities, it has not previously been identified as a melanogenesis inhibitor. In this report we describe the inhibitory effects of biochanin A on melanogenesis under both *in vitro* and *in vivo* conditions.

Materials and Methods

Chemicals and antibodies. Triton X-100, phenylmethylsulfonyl fluoride (PMSF), L-DOPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), arbutin, dimethyl sulfoxide (DMSO), trypsin/EDTA, synthetic melanin, 1-phenyl-2-thiourea (PTU), and 3-isobutyl-1-methylxanthin (IBMX) were purchased from Sigma (St. Louis, MO). All other chemicals, including formononetin, were from Tokyo Chemical Industry (Tokyo), and were of analytic reagent grade.

Isolation of biochanin A from T. pratense. Dried leaves and stems of *T. pratense* (1.0 kg) were sequentially macerated with *n*-hexane (5 × 4 L), EtOAc (5 × 4 L), and EtOH (5 × 4 L) to yield *n*-hexane, EtOAc, and EtOH extracts upon evaporation. Gradient elution of the EtOAc extract of the leaf and stem on a silica gel column using *n*-hexane-acetone (4:1 → 2:3) as the mobile phase yielded eight fractions (TP1–TP8). Fraction TP4 was further purified on two successive silica gel columns by elution with ethyl acetate, followed by a gradient mixture of *n*-hexane-acetone (4:1 → 3:2), yielding biochanin A (16.4 mg).

† To whom correspondence should be addressed. Tel: +886-6-2602137; Fax: +886-6-2602137; E-mail: mozyme2001@gmail.com
* These two authors contributed equally to this work.

Biochanin A: Yellow needles; mp 214–215 °C; ESI/MS *m/z*: 285 $[M+H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 3.86 (3H, s, COC$\underline{H}_3$), 6.25 (1H, *d*, *J* = 2.0 Hz, H-6), 6.43 (1H, *d*, *J* = 2.0 Hz, H-8), 7.01 (2H, *d*, *J* = 8.6 Hz, H-3′, 5′), 7.50 (2H, *d*, *J* = 8.6 Hz, H-2′, 6′), 8.37 (1H, s, H-2); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 55.6 (O$\underline{C}H_3$), 94.0 (C-8), 98.6 (C-6), 104.4 (C-10), 113.5 (3′, 5′), 121.8 (C-1), 122.6 (C-3), 130.3 (C-2′, 6′), 154.6 (C-2), 157.8 (C-9), 161.4 (C-5), 164.1 (C-7), 179.7 (C-4). These data were compared with published values.[10]

Cell cultures and drug treatments. Mouse B16 melanoma cells (4A5) were purchased from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal bovine serum at 37 °C in a humidified, CO_2-controlled (5%) incubator. The cells were seeded at an appropriate cell density in a 24-well. After 1 d of incubation, the cells were treated with various concentrations of the drug in the absence and the presence of a stimulatory agent (100 µM IBMX) for a further 16 h (for tyrosinase activity assay) or 48 h (for melanin determination). They were harvested at the designated times and used in the assays described.

Measurement of cell viability. An MTT assay was performed to examine the viability of the cells. After the cells were incubated with the drug for 48 h, the culture medium was removed and replaced with 1 mg/mL of MTT solution dissolved in phosphate-buffered saline (PBS), and the mixture incubated for an additional 2 h. The MTT solution was then removed, DMSO was added, and the absorbance of the dissolved formazan crystals was determined at 570 nm with a spectrophotometer.

Determination of melanin content. At the end of cell culture, the cells were washed twice with PBS and lysed in lysis buffer containing 20 mM sodium phosphate (pH 6.8) and 1% Triton X-100. After centrifugation at 15,000 × *g* for 15 min, the pellets were placed in 1 N NaOH containing 20% DMSO for 1 h at 95 °C to dissolve the melanin. The absorbance at 490 nm was measured, and the melanin content was measured against an authentic standard of synthetic melanin. The protein content in the supernatant was determined by Bradford assay with bovine serum albumin (BSA) as the protein standard. The specific melanin content was adjusted by the amount of protein in the same reaction.

Measurement of cellular tyrosinase activity. Tyrosinase activity in the B16 cells was examined by measuring the rate of oxidation of L-DOPA.[11] The drug-treated cells were washed with ice-cold PBS and lysed with 20 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 and 1 mM PMSF. Detergent was used to release membrane-bound tyrosinase from the melanosomes. The cells were then disrupted by freezing and thawing. The lysates were centrifuged at 15,000 × *g* for 15 min. The protein content in the supernatant was determined by Bradford assay, with BSA as the protein standard. Tyrosinase activity was then determined as follows: one mL of the reaction mixture contained 50 mM of phosphate buffer (pH 6.8), 2.5 mM of L-DOPA, and 500 µg of the supernatant protein. After 15 min of reaction at 37 °C, dopachrome formation was monitored by measuring the absorbance at 475 nm. One unit of tyrosinase activity was defined as the amount of enzyme protein that catalyzes the formation of 1 µ mole of dopachrome in 1 min. The amount of dopachrome in the reaction was calculated by the Lambert-Beer Law using a molar extinction coefficient for dopachrome of 3,600 $M^{-1}\cdot cm^{-1}$.[12] Specific tyrosinase activity was normalized by the protein content in the reaction.

Tyrosinase zymography. L-DOPA staining assay was performed as reported by Miyake *et al.*[11] The cells were washed 3 times in ice-cold PBS and lysed in cold lysis buffer (20 mM sodium phosphate pH 6.8, 1% Triton X-100, and 1 mM PMSF) containing a protease inhibitor cocktail (Abcam, Cambridge, UK). An aliquot of the lysate was used to determine the protein content by Bradford assay using BSA as standard. The proteins (100 µg) were mixed with sampling buffer (no β-mercaptoethanol or heating) and separated by 10% SDS-polyacrylamide gel electrophoresis. The gel was rinsed in 200 mL of 100 mM sodium phosphate buffer (pH 6.8) and equilibrated at room temperature with gentle shaking. After 30 min, the rinse buffer was replaced with fresh buffer. After repetition of the rinse procedure, the gel was transferred to 200 mL of a staining solution containing the rinse buffer supplemented with 5 mM L-DOPA, and the mixture was incubated in the dark at 37 °C for 1 h. Tyrosinase activity was visualized in the gel as a dark melanin-containing band. The signal intensity of each band was quantified with a densitometer system GS-700 (Bio-Rad, Hercules) equipped with an integrator.

Determination of depigmenting activity in zebrafish. Depigmenting activity in the zebrafish model system was assayed following Choi *et al.*[13] The study was approved by the Ethics Committee of the National University of Tainan (approval number 9804). Adult zebrafish were obtained from a commercial dealer and kept in acrylic tanks under a 14/10 h light/dark cycle at 28 °C. Synchronized embryos were obtained from natural spawning induced in the morning by turning on a light. Test compounds were dissolved in 0.1% DMSO and then added to the embryo medium from 9 to 48 hpf (hours post-fertilization). The developed zebrafish larvae were sonicated in cold lysis buffer (20 mM sodium phosphate pH 6.8, 1% Triton X-100, and 1 mM PMSF) containing a protease inhibitor cocktail. An aliquot of the lysate was used to determine the protein content by Bradford assay using BSA as standard. The lysate was clarified by centrifugation at 10,000 × *g* for 10 min. The melanin precipitate of the various tubes was photographed with a digital camera and then resuspended with 0.2 mL of 1 N NaOH/20% DMSO at 95 °C for 1 h. The absorbance at 490 nm was measured, and the melanin content was quantified against an authentic standard of synthetic melanin. The specific melanin content was adjusted by the amount of protein in the same reaction.

In the tyrosinase activity assay, 250 µg of total protein in the lysate was added to a reaction mixture containing 50 mM phosphate buffer pH 6.8 and 2.5 mM L-DOPA. After incubation at 37 °C for 60 min, dopachrome formation was monitored by measuring the absorbance at 475 nm. Specific tyrosinase activity was normalized by the protein in the reaction, as described above.

Determination of depigmenting activity in mice. Depigmenting activity was evaluated in a mouse model system using a modification of the protocol of Tai *et al.*[14] The study was approved by the Institutional Animal Care and Use Committee of National Formosa University (approval number A99-03). Six 5-week-old mice (C57BL/6J), weighing approximately 20 to 25 g, were obtained from the National Animal Laboratory Center (Taipei, Taiwan). Throughout the experiment, the animals were housed in stainless steel cages in an air-conditioned room, temperature maintained at 25 to 28 °C under a day-night cycle of 12 h. The animals were acclimatized for 7 d prior to the experiment. After shaving of their hair, the animals were given 1 d of rest, and then the right side of the back skin was smeared only with 0.1 g of vaseline (control), while the left side was smeared with 0.1 g of 2% biochanin A in vaseline. Application continued twice daily for 3 weeks, and the skin-whitening index value (L value) was measured on the same skin area at 3-d intervals with a Chromameter CR-2300d (Konica Minolta, Osaka). This is a colorimetric instrument that uses a xenon lamp as light source and is connected to a computer. The light reflected perpendicularly from the skin was collected by photodetectors with colored filters for tri-stimulus color analysis at 450, 560, and 600 nm, using the L*a*b* system, following the CIE color system.[15] We considered only the L parameter. The L-value gives relative brightness, ranging from total black (L = 0) to total white (L = 100). After calibration, an 8-mm probe was applied to the skin simply by the weight of the instrument. After the shutter button was pushed, the results were immediately read on the monitor. The initial skin-whitening index L-value was taken from the skin of each side of the mouse, prior to the next application of the tested substances. During measurement, the photoreceivers were placed perpendicularly to the skin with minimal pressure.

Statistical analysis. All of the data in the present study were obtained as averages of experiments that were performed at least in triplicate, and are expressed as means ± SD. Statistical analysis was done by Student's *t* test. A value of $p < 0.05$ (*) or $p < 0.001$ (**) was considered statistically significant.

Results and Discussion

Isolation and identification of biochanin A from T. pratense

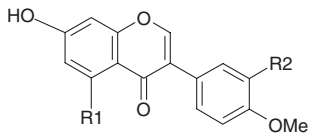
In our preliminary study, a methanol extract of *T. pratense* showed strong inhibitory activity against melanogenesis in B16 cells. Following bioassay-guided purification of the extract by methanol extraction, *n*-hexane, ethyl acetate, *n*-BuOH, and water partitioning, and repeated silica gel column chromatography, one active compound was ultimately isolated. The chemical structure of this compound was determined by mass spectra and ^{1}H-NMR, and ^{13}C-NMR analysis, and the structure was resolved by comparing these data with those in the literature.[10] The purified compound was identified as biochanin A (Fig. 1).

Evaluation of the depigmenting activity of biochanin A in vitro

The depigmenting activity of biochanin A was first evaluated in cultured B16 cells *in vitro*. Figure 2 shows the cytotoxicity of the compound toward the cells. As shown in the figure, 22 μM biochanin A had no significant cytotoxic effects on the cells, and hence this was used as the maximal concentration in the *in vitro* depigmenting assay. The melanin content of the biochanin A-treated cells was used as a measure of anti-melanogenic activity. In the present study, we used IBMX, an agent that stimulates intracellular cAMP levels, to stimulate melanogenesis in the B16 cells. As shown in Fig. 3A, the melanin content of the B16 cells increased considerably after stimulation with IBMX. Biochanin A treatment resulted in significant prevention of the increase in melanin content induced by IBMX in the B16 cells (Fig. 3A). This inhibition of melanogenesis was dose-dependent. When the inhibitory potencies of biochanin A and arbutin were compared, 22 μM biochanin A exhibited anti-melanogenic activity comparable to that of 400 μM arbutin.

Biochanin A treatment also resulted in a decrease in cellular tyrosinase activity. Tyrosinase is the key enzyme in melanogenesis. As shown in Fig. 3B, biochanin A treatment reduced the amounts of cellular tyrosinase activity recovered in a dose-dependent manner. The reduction of cellular tyrosinase activity due to biochanin A was thought to be attributable either to direct inhibition of tyrosinase activity or to repression of tyrosinase gene expression. However, the latter possibility was excluded by zymographical examination of the level of active tyrosinase protein in the B16 cells (Fig. 4), where the amounts of active tyrosinase in the biochanin A-treated cells were not significantly different from that in the IBMX-stimulated control cells. Thus biochanin A appeared to inhibit melanogenesis in B16 cells by directly inhibiting the cellular activities of tyrosinase.

Another isoflavone, calycosin (4′-methoxy-3′,7-dihydroxyisoflavone), with structural similarity to biochanin A (4′-methoxy-5,7-dihydroxyisoflavone), has also been reported to show both anti-melanogenic and anti-tyrosinase activities in mouse melanoma cells.[16] It is well



Biochanin A, R1 = OH, R2 = H
Calycosin, R1 = H, R2 = OH
Formononetin, R1 = R2 = H

Fig. 1. Chemical Structures of Biochanin A, Calycosin, and Formononetin.

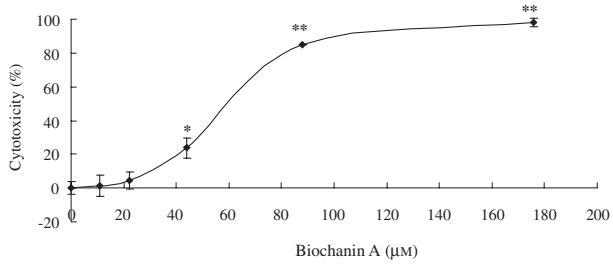


Fig. 2. Cytotoxicity of Biochanin A toward B16 Cells.
The cells were seeded in 24-well plates for 1 d and then treated with various dosages of biochanin A (176–0 μM) in the presence of 100 μM of IBMX for 2 d. Cell viability was then examined by the MTT assay. Averaged data (n = 3) are presented with an error bar of SD. A value of $p < 0.05$ (*) or $p < 0.001$ (**) by Student's *t*-test by comparing the data with that at 0 μM biochanin A was considered statistically significant.

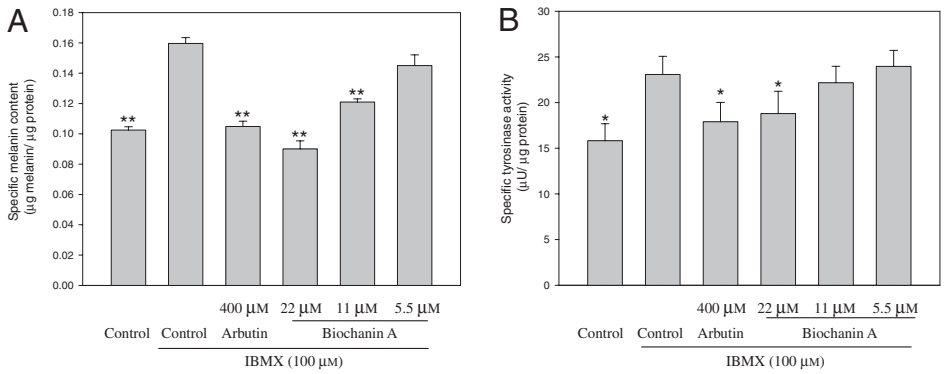


Fig. 3. Effects of Biochanin A on Melanogenesis (A) and Cellular Tyrosinase Activity (B) in B16 Cells.
The cells were cultivated for 1 d and then stimulated with 100 μM of IBMX for 2 d in various concentrations of biochanin A. The melanin, cellular tyrosinase activity, and protein contents of the cells were determined by spectrometry, as described in Experimental, and the specific melanin and cellular tyrosinase activity contents of each reaction were calculated by dividing the melanin and tyrosinase activity contents by the protein content in the same reaction. Averaged data (n = 3) are presented with an error bar of SD. A value of $p < 0.05$ (*) or $p < 0.001$ (**) by Student's *t*-test by comparing the data with those for the IBMX-stimulated control were considered statistically significant.

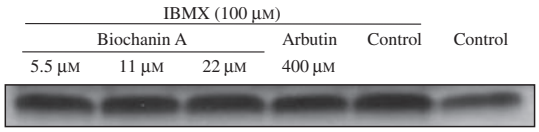


Fig. 4. Zymography of Tyrosinase Activity in B16 Cells Treated with Biochanin A.

Cells were inoculated in 24-well plates for 1 d and then stimulated with 100 μM of IBMX with and without the test drug. They were harvested, and tyrosinase activity was analyzed by zymography of in-gel L-DOPA reactivity after electrophoresis, as described in Experimental.

known that the numbers and positions of the functional groups on the skeleton of isoflavones strongly affect their bioactivity. Based on structure/activity relationships (SAR), another commercial isoflavone, formononetin (4′-methoxy-7-hydroxyisoflavone), which contains the same 7-OH and 4′-OMe functional groups as biochanin A and calycosin in the isoflavone skeleton, was selected to test its ability to inhibit melanogenesis in B16 cells. The results are shown in Fig. 5. Formononetin inhibited melanogenesis in the cells in a dose-dependent manner analogous to that seen for biochanin A (Fig. 5A), without

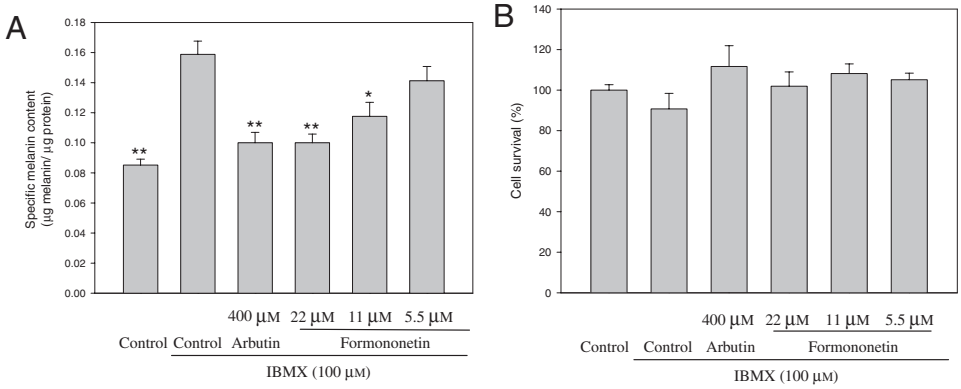


Fig. 5. Effects of Formononetin on Melanogenesis (A) and Cell Survival (B) in B16 Cells.

Cells were seeded in 24-well plates for 1 d and then treated with various dosages of formononetin (22–0 μM) for 2 d. Melanin contents and cell viability were then examined. Averaged data (n = 3) are presented with an error bar of SD. A value of $p < 0.05$ (*) or $p < 0.001$ (**) by Student's *t*-test by comparing the data with those for the IBMX-stimulated control was considered statistically significant.

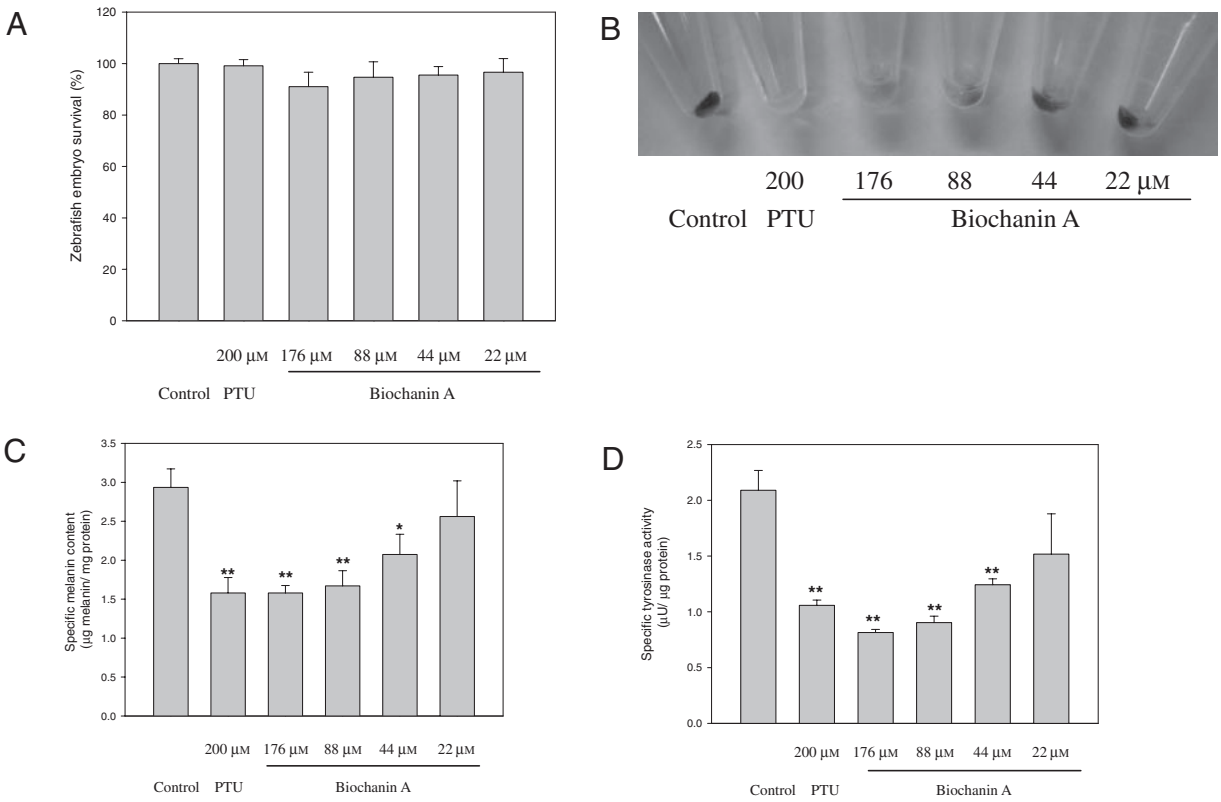


Fig. 6. Evaluation of Depigmenting Activity of Biochanin A in Zebrafish.

A total of 100 synchronized embryos were treated with drugs from 9 to 48 hpf, and normally developed embryos were collected to calculate embryo survival (A). After sonication and centrifugation, melanin pigment was photographed (B) and its quantity was determined by a photometric method (C), as described in Experimental. For assays of tyrosinase activity (D), 250 μg of total protein from lysates of 100 zebrafish larval was incubated with 2.5 mM of L-DOPA, and the resulting dopachrome was quantified by a photometric method, as described in Experimental. Averaged data (n = 3) are presented with an error bar of SD. A value of $p < 0.05$ (*) or $p < 0.001$ (**) by Student's *t*-test by comparing the data with those for control was considered statistically significant.

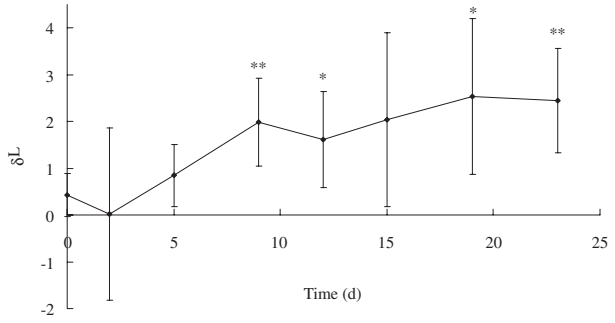


Fig. 7. Evaluation of Depigmenting Activity of Biochanin A in Mice.
Six mice were treated with vaseline only (right side) or 2% biochanin A in vaseline (left) on the skin of the backside for 3 weeks. The skin-whitening index value (L value) of the skin on each side of each mouse's back was recorded at 3-d intervals, and the δL value between the two sides was then calculated for the various intervals. The average δL value ($n = 6$) with an error bar of SD was plotted over the experimental period. A value of $p < 0.05$ (*) or $p < 0.001$ (**) by Student's *t*-test by comparing the data with those at time zero was considered statistically significant.

apparent cytotoxicity at the tested concentrations (Fig. 5B). This suggests that the formononetin-based structure plays a key role in melanogenesis inhibition. However, due to insufficient data, a more detail SAR study is needed to confirm the exact situation.

Evaluation of the in vivo *depigmenting activity of biochanin A*

The depigmenting activity of biochanin A was evaluated by two *in vivo* systems, zebrafish and mice, in order to examine the effectiveness of this newly discovered compound as a potential human therapeutic drug. Zebrafish have recently been established as a new *in vivo* model for evaluating the depigmenting activity of melanogenic regulatory compounds.[13] This animal model system has advantages, including easy maintenance and handling of the animals, and high efficiency in drug penetration through the skin. For these reasons, the zebrafish model was used as an *in vivo* system to evaluate the inhibition of melanogenesis by biochanin A. As shown in Fig. 6, biochanin A showed no significant toxicity towards zebrafish embryos at the tested concentrations, up to 176 μM (Fig. 6A). However, treatment of the embryos with biochanin A between 9 to 48 pfh significantly reduced skin melanin in the developed larvae (Fig. 6B and C). At a concentration of 176 μM, biochanin A caused the same degree of transparency as seen for 200 μM of PTU, a standard depigmenting agent used in zebrafish experiments. The specific melanin content of the biochanin A-treated zebrafish decreased dose-dependently as compared to that of the untreated fish. The reduction in cellular tyrosinase due to biochanin A was also dose-dependent (Fig. 6D), indicating that biochanin A reduced melanogenesis in the zebrafish by suppression of cellular tyrosinase activity.

Because the skin of mice is more like that of humans than that of zebrafish, we also used mice as a second *in vivo* animal model to investigate the depigmenting activity of biochanin A. As shown in Fig. 7, the differences in the skin-whitening index values of skin color (δL values) between 2% biochanin A treatment and the placebo control significantly increased after 1 week of treatment, and the increase continued to the end of the experiment. Hence, biochanin A is promising as a depigmenting agent for mammalian skin.

Recently, many isoflavonoids have been identified as potent tyrosinase inhibitors.[2,3,16–20] Some of these have been found to have inhibitory activity on melanogenesis in *in vitro* cell-culture models.[17–19] Compounds such as 8-hydroxydaidzein[14] and haginin A[17] were also found to have strong skin *in vivo* depigmenting activity. In the present study, the isoflavone biochanin A inhibited melanogenesis not only *in vitro* in cultured B16 melanoma cells but also *in vivo* in the skin of zebrafish embryos and mice. In conclusion, our results indicate that biochanin A is an effective melanogenesis inhibitor and has potential as an applied agent for the therapeutic treatment of skin hyperpigmentation disorders.

Acknowledgments

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References

1) Chang TS, *Int. J. Mol. Sci.*, **10**, 2440–2475 (2009).
2) Chang TS, *J. Agric. Food Chem.*, **55**, 2010–2015 (2007).
3) Chang TS, Ding HY, Tai SS, and Wu CY, *Food Chem.*, **105**, 1430–1438 (2007).
4) Ding HY, Lin HC, and Chang TS, *J. Cosmet. Sci.*, **60**, 347–352 (2009).
5) Chae YH, Coffing SL, Cook VM, Ho DK, Cassady JM, and Baird WM, *Carcinogenesis*, **12**, 2001–2006 (1991).
6) DuBois JL and Sneden AT, *J. Nat. Prod.*, **58**, 629–632 (1995).
7) Shen P, Liu MH, Ng TY, Chan YH, and Yong EL, *J. Nutr.*, **136**, 899–905 (2006).
8) Kim JM and Yun-Choi HS, *Arch. Pharm. Res.*, **31**, 886–890 (2008).
9) Sartorelli P, Carvalho CS, Reimão JQ, Ferreira MJ, and Tempone AG, *Parasitol. Res.*, **104**, 311–314 (2009).
10) Chang YC, Nair MG, Santell RC, and Helferich WG, *J. Agric. Food Chem.*, **42**, 1869–1871 (1994).
11) Miyake M, Yamamoto S, Sano O, Fuji M, Kohno K, Ushio S, Iwaki K, and Fukuda S, *Biosci. Biotechnol. Biochem.*, **74**, 753–758 (2010).
12) Neeley E, Fritch G, Fuller A, Wolfe J, Wright J, and Flurkey W, *Int. J. Mol. Sci.*, **10**, 3811–3823 (2009).
13) Choi TY, Kim JH, Ko DH, Kim CH, Hwang JS, Ahn S, Kim SY, Kim CD, Lee JH, and Yoon TJ, *Pigment Cell Res.*, **20**, 120–127 (2007).
14) Tai SS, Lin CG, Wu MH, and Chang TS, *Int. J. Mol. Sci.*, **10**, 4257–4266 (2009).
15) Takiwaki H and Serup J, *Skin Pharmacol.*, **7**, 145–150 (1994).
16) Kim JH, Kim MR, Lee ES, and Lee CH, *Biol. Pharm. Bull.*, **32**, 264–268 (2009).
17) Kim JH, Baek SH, Kim DH, Choi TY, Yoon TJ, Hwang JS, Kim MR, Kwon HJ, and Lee CH, *J. Invest. Dermatol.*, **128**, 1227–1235 (2008).
18) Kim HJ, Seo SH, Lee BG, and Lee YS, *Planta Med.*, **71**, 785–787 (2005).
19) Lee MH, Lin YP, Hsu FL, Zhana GR, and Yen KY, *Phytochemistry*, **67**, 1262–1270 (2006).
20) Kim JM, Ko RK, Jung DS, Kim SS, and Lee NH, *Phytother. Res.*, **24**, 70–75 (2010).