Suppression of Melanin Synthesis by the Phenolic Constituents of Sappanwood (Caesalpinia sappan)

Kaoru Mitani1, Fumihide Takano1,2, Tetsuro Kawabata1, Ahmed E. Allam1, Mayumi Ota1, Tomoya Takahashi1, Nobuo Yahagi1, Chikai Sakurada2, Shinji Fushiya2, Tomihisa Ohta1

Affiliations
1 Pharmacognosy and Chemistry of Natural Products, School of Pharmacy and Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan
2 Nihon Pharmaceutical University, Saitama, Japan

Abstract

Sappanwood (Caesalpinia sappan Linn.) is used as an herbal medicine. It is sometimes used to treat skin damage or as a facial cleanser. In the present study, the methanol (MeOH) extract of sappanwood was found to inhibit melanin synthesis in cultured human melanoma HMV-II cells stimulated with forskolin, and six active compounds (1–5 and 7) were isolated from the extract along with a non-active compound (6). Compounds 2–7 were identified as sappanchalcone (2), 3′-deoxy-4-O-methylsappanol (3), brazilin (4), brazilin (5), sappanol (6), and 4-O-methylsappanol (7). Compound 1 was a new compound, and its structure was determined to be (6αS,11bR)-7,11b-dihydro-6H-indeno[2,1-c]chromene-3,6α,10,11-tetrol by spectroscopic analyses. Among the six active compounds, brazilin (5) (EC50: 3.0 ± 0.5 µM) and 4-O-methylsappanol (7) (EC50: 4.6 ± 0.7 µM) strongly suppressed melanin synthesis in HMV-II cells. Bioactive compounds showed moderate cytotoxicities against HMV-II cells with IC50 values of 83.1 ± 4.0 µM (for 2), 72.0 µM ± 2.4 (for 3), 33.8 ± 1.1 µM (for 4), 18.4 ± 0.8 µM (for 5), and 20.2 ± 0.8 (for 7), respectively. Brazilin (5) selectively suppressed the expression of mRNAs for tyrosinase-related protein (TYRP) 2 and tyrosinase but did not influence the expression of TYRP1. These results suggest that brazilin (5) is a new class of melanin inhibitor and that sappanwood could be used as a cosmetic material.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Melanin is a biopigment synthesized in melanosomes. Melanosomes enclosing melanin polymers are transferred from melanocytes to surrounding keratinocytes in the human epidermis. The color of human skin is determined primarily by the quantity, type, and distribution of melanin in keratinocytes [1,2]. Melanin has a critical role in the protection of the skin from solar ultraviolet (UV) radiation [3], but excess synthesis of melanin leads to hyperpigmentation disorders such as melasma, lentigo, and age spots [4]. Hence, the inhibitors of melanin biosynthesis have received considerable attention in clinical and cosmetic research, and melanin inhibitors such as hydroquinone [5], kojic acid [6,7], arbutin [8,9], and linoletic acid [10,11] have been discovered.

“Sappan Lignum” is the heartwood of sappanwood (Caesalpinia sappan Linn.; Fabaceae) and is used both as a dyestuff and as an herbal medicine [12]. It has been used to treat wounds, skin diseases, leprosy, dysentery, menorrhagia, menoxenia, leukorrhea, and diabetic complications [13]. Several pharmacological studies have been undertaken focusing on the extracts and constituents of this plant as well as on the various activities found (e.g., immunomodulatory, [14], anti-inflammatory [15], antioxidant [16], hypoglycemic [17], vasorelaxant [12], spasmylolytic [18], and anti-arthritic [19] effects). However, the effect on melanin synthesis of the extracts and constituents of this plant has not been studied in detail. In the present study, we investigated the inhibitory effects of the methanol (MeOH) extract of sappanwood on melanin synthesis in human melanoma HMV-II cells stimulated with forskolin. The MeOH extract showed inhibitory activity, and six compounds (one of which is novel) were isolated as active principles from the extract. Furthermore, the mechanism of action for the most potent inhibitory compound, brazilin 5, was investigated.
Materials and Methods

General

Values for optical rotation were determined with a Horiba SEPA-3000 high-sensitivity polarimeter (Horiba). UV data were obtained with a Shimadzu UV-1600 UV–visible spectrometer (Shimadzu). IR data were obtained with a Shimadzu IR-460 IR spectrophotometer. Circular dichroism (CD) data were obtained using a JASCO J-820 spectrometer (JASCO).

NMR spectra were recorded with a JEOL GSX-500 spectrometer (JEOL) in acetone-d$_6$ and dimethyl sulfoxide (DMSO)-d$_6$. Chemical shifts were referenced to the residual solvent peaks acetone-d$_6$ (δ$_H$ 2.04 and δ$_C$ 29.8) and DMSO-d$_6$ (δ$_H$ 2.49 and δ$_C$ 39.5). Mass spectra were measured on a JEOL SX-102 mass spectrometer. Reversed-phase high-performance liquid chromatography (HPLC) was performed on an ODS column (particle size: 5 µm, TOSO, 18 mm × 250 mm). Silica gel (63–210 µm; Kanto Kagaku) and ODS (63–212 µm; Wako Pure Chemical Industries) were used for open-column chromatography. Thin-layer chromatography (TLC) was done on silica gel 60 F$_{254}$ (Merck) and RP-18 F$_{254s}$ (Merck).

Plant material

_C. sappan_ for medicinal use (lot number: 313116) was purchased from Uchida Wakanyaku Co., Ltd. and identified by the authors. A voucher specimen of the plant material (T-232) was deposited in the Laboratory of the Pharmacognosy and Chemistry of Natural Products, School of Pharmacy and Pharmaceutical Sciences, Kanazawa University (Kanazawa, Japan).

Extraction and isolation

The air-dried heartwood of _C. sappan_ (5.0 kg) was extracted thrice with MeOH (3 L) at room temperature. The MeOH solution (9 L) was passed through filter paper (Advantec MFS, Inc.), and the solvent removed under reduced pressure at 35°C to yield the MeOH extract (408 g). The MeOH extract (390 g) was suspended in H$_2$O and partitioned successively with n-hexane (1 L × 3), ethyl acetate (EtOAc) (1 L × 5), and n-butanol (n-BuOH) (1 L × 3), respectively, to give n-hexane (9.46 g), EtOAc (345 g), n-BuOH (17.4 g), and H$_2$O (22.7 g) fractions.

The EtOAc fraction was further separated by column chromatography (80 mm × 200 mm) on SiO$_2$, eluted with a gradient of n-hexane and EtOAc (n-hexane : EtOAc = 5:5 → 4:6 → 2:8 → EtOAc) and MeOH to afford six fractions. The third fraction (54.0 g) eluted by n-hexane : EtOAc = 2:8) was subjected to additional column chromatography on SiO$_2$ and eluted with a gradient of chloroform and methanol (CHCl$_3$ : MeOH = 14:1 → 9:1 → 7:1 → 5:1 → MeOH) to give nine fractions. A part of the fraction eluted by CHCl$_3$ : MeOH = 14:1 (20 mg) was purified by HPLC on a SiO$_2$ column (TOSO; 18 mm × 250 mm; particle size, 5 µm; flow rate, 5 ml/min) with the eluting solvent (CHCl$_3$ : MeOH = 97:3) to give compound 2 (6.04 mg; purity > 91%). A further portion of the same fraction (80 mg) was also separated by HPLC on a SiO$_2$ column with the eluting solvent (CHCl$_3$ : MeOH = 14:1) to give compound 3 (2.21 mg; purity > 90%). A further portion of the same fraction (850 mg) was subjected to ODS column (80 mm × 200 mm) chromatography with elution with a gradient of aqueous acetonitrile solution (10% → 25% → 40% → 70% → 100%) to give seven fractions. The fraction eluted by 25% aqueous acetonitrile was purified by HPLC on an ODS column by elution with the same aqueous acetonitrile solvent to afford compound 4 (1.79 mg; purity > 90%). The fraction eluted by CHCl$_3$ : MeOH (14:1) (3.7 g) was separated further by HPLC on a preparative ODS column with the eluting aqueous acetonitrile solvent system (10% → 25% → 40% → 100%). Compound 5 (2.99 g; purity > 90%) was purified from the fraction eluted by 10% aqueous acetonitrile. Compounds 6 (3.97 mg; purity = 92%) and 7 (16.0 mg; purity > 92%) were separated from the fraction eluted by 25% aqueous acetonitrile. A new compound 1 (15 mg; purity > 93%) was obtained from the fraction eluted by 40% aqueous acetonitrile.

Compounds 2–7 were isolated and identified as sappanchalcone (2), 3′-deoxy-4-O-methylsappanol (3), brazilein (4), brazilin (5), sappanol (6), and 4-O-methylsappanol (7) by comparison of their spectral data with those reported in the literature [20–24].

Measurement of melanin content in cultured human melanoma HMV-II cells

Intracellular and extracellular melanin contents in cultured HMV-II human melanoma cells (Dainippon Pharmaceutical Co.) were determined according to methods described previously with minor modification [25–28]. Cells were passed once a week and used between passages 5 and 8. Briefly, cultured HMV-II cells were trypsinized [0.25% trypsin and 0.1% ethylenediamine tetra-acetic acid (EDTA) at 37°C for 5–10 min]. Cells [2.5 × 10^6 cells/well in 980 µl Dulbecco’s modified Eagle’s medium (DMEM)] were inoculated with a pipette into 24-well plates and incubated for 24 h at 37°C in a CO$_2$ incubator. After 24-h incubation, 20 µl of sample solution was added to each well, and the 24-well plate incubated for 2 days at 37°C in a CO$_2$ incubator. Each sample was tested in triplicate. Test samples were dissolved in dimethyl sulfoxide/ phosphate-buffered saline (DMSO/PBS) and then diluted with DMEM to achieve the appropriate concentrations. The final concentration of DMSO was 0.03%. In the control group, DMEM/PBS (1:1, v/v) solution diluted with DMEM to 0.03% of the final DMSO concentration was used instead of the sample solution.

We used α-arbutin (purity ≥ 98%; Sigma-Aldrich) or kojic acid (purity = 98.0%; Sigma-Aldrich) as the reference compounds for the inhibition of intracellular and extracellular melanogenesis. HMV-II cells were incubated with tested samples, reference compounds, or vehicle in the presence or absence of phorbol-12-myristate-13-acetate (PMA; an activator of protein kinase C) or forskolin (an activator of adenylate cyclase) [29] for 120 h. At the indicated time points, the culture medium was removed with a 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 5–10 min) and washed with 100 µl PBS. Cells were digested by the addition of 400 µl 1 N NaOH and left to stand for 16 h at room temperature. The optical density (OD) of the resulting solution was measured at 475 nm, and the amount of intracellular melanin calculated. The culture medium was centrifuged (600 × g for 10 min at 4°C). An aliquot of the supernatant (1 ml) was added to 1 ml of a mixture of 0.4 M 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 6.8) and ethanol (9:1; v/v). The OD of the resulting solution was measured at 475 nm. The amount of melanin retained in the cells [intracellular melanin] and that secreted into cultured medium [extracellular melanin] were determined separately as described by Kawabata et al. [26].

Mitani K et al. Suppression of Melanin... Planta Med 2013; 79: 37–44
Cytotoxicity assay
To determine the cytotoxicity of the tested samples, HMV-II cells (180 µL) were seeded on 96-well plates at 1.0 × 10^5 cells per well with tested samples (purity > 93%; 20 µL in DMSO/PBS) at various concentrations. After 48-h cultivation, supernatants were removed, and adherent (B16F1) or non-adherent (THP-1) cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 10 µL, 5 mg/mL in PBS) for 4 h and then solubilized with 10% (v/v) sodium dodecyl sulfate (SDS; in 60% (v/v) dimethyl formamide) solution (100 µL) for 10 h. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Model 550). Cell viability was calculated by comparing the absorbance with that of the non-treated control culture. A cell growth curve was plotted. Values for the half-maximal inhibitory concentration (IC50) were calculated using simple linear regression.

Table 1  1H and 13C NMR data for 1a.

<table>
<thead>
<tr>
<th>Position</th>
<th>δ_C</th>
<th>δ_H (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>132.8</td>
<td>CH</td>
<td>7.72 d (8.2)</td>
</tr>
<tr>
<td>2</td>
<td>110.2</td>
<td>CH</td>
<td>6.35 dd (2.0, 8.9)</td>
</tr>
<tr>
<td>3</td>
<td>157.4</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>103.6</td>
<td>CH</td>
<td>6.25 d (2.0)</td>
</tr>
<tr>
<td>4a</td>
<td>155.0</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>70.4</td>
<td>CH2</td>
<td>4.08 d (10.3), 3.85 d (11.0)</td>
</tr>
<tr>
<td>6a</td>
<td>79.4</td>
<td>qC</td>
<td>-OH 5.05b</td>
</tr>
<tr>
<td>7</td>
<td>41.7</td>
<td>CH2</td>
<td>3.21 d (15.8), 2.68 d (15.8)</td>
</tr>
<tr>
<td>7a</td>
<td>134.6</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>116.5</td>
<td>CH</td>
<td>6.47 d (8.2)</td>
</tr>
<tr>
<td>9</td>
<td>114.8</td>
<td>CH</td>
<td>6.62 d (8.5)</td>
</tr>
<tr>
<td>10</td>
<td>144.2</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>143.3</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>11a</td>
<td>132.5</td>
<td>qC</td>
<td></td>
</tr>
<tr>
<td>11b</td>
<td>51.1</td>
<td>CH</td>
<td>4.29 s</td>
</tr>
<tr>
<td>11c</td>
<td>117.8</td>
<td>qC</td>
<td></td>
</tr>
</tbody>
</table>

*1H and 13C NMR for 1 were measured in aceton-d_6, b For detection of the hydroxyl signal of position 6a; 1H NMR for 1 was measured in DMSO-d_6
Reverse transcription-polymerase chain reaction (RT-PCR)

Total ribonucleic acid (RNA) was prepared directly from HMV-II cells administered with samples for 8 h using a commercially available kit (RNaseasy Mini Kit; Qiagen GmbH). Isolated RNA was quantified by photometry at 260 nm/280 nm. The quality of RNA was determined by measuring the ratio of 18S/28S ribosomal band intensity in an ethidium bromide containing 1.5% agarose gel after electrophoresis. A PrimerScript One-step RT-PCR kit ver 2 (Takara Bio, Inc.) was used for preparing complementary deoxyribonucleic acid (cDNA). First-strand cDNAs were synthesized by one cycle of reverse transcription at 50 °C for 30 min and one cycle of reverse transcriptase incubated at 94 °C for 2 min. Second-strand cDNAs synthesis and PCR amplification for tyrosinase as well as tyrosinase-related proteins 1 and 2 (TYRP1 and TYRP2, respectively) were carried out at 94°C for 30 s, 53–57°C for 30 s, and 72°C for 1 min. For each combination of primers, the kinetics of the PCR amplification was determined beforehand and semi-quantitative PCR undertaken in the exponential range. The β-actin (ACT) gene was used as an endogenous internal standard and amplified with specific primers simultaneously. PCR products were subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide staining under UV irradiation. The primer sequences used were: TYR forward, 5'-AGGCAGAGGTTCCTGTCAGA-3' and TYR reverse, 5'-ATTGTGTCTCGTGCTTTGAG-3'; TYRP1 forward, 5'-CATGATGGCGAGAGATGACG-3' and TYRP1 reverse: 5'-TCTGTGAAGGTGTGCAGGAG-3'; TYRP2 forward, 5'-AGTGGATTCGGCACGAACATCC-3' and TYRP2 reverse, 5'-CTGGAGGAAGAAAGGAACC-3'; ACTB forward, 5'-AGACGTACAGGCTCTGGAC-3' and ACT reverse, 5'-AGCCTGTGGTGCGTACAG-3'.

Data analyses

Mean melanin production (µg/mL) and the percentage of control of melanogenesis are reported as the mean ± SD of two or three independent experiments (triplicate/one experiment). Statistical significance was determined by Dunnett’s multiple test after one-way analysis of variance (ANOVA) with comparison to a control group. p < 0.05 was considered statistically significant.

Supporting information

Spectroscopic data for compound 1 are available as Supporting Information.

Results

In the present study, we used human melanoma HMV-II cells instead of the previously used murine melanoma B16F1 cells [26] to assess the inhibitory effect on melanin synthesis. The potential of human melanoma HMV-II cells to synthesize melanin was therefore investigated. Melanin contents in cultured HMV-II cells increased depending upon the concentration of the stimulants forskolin, an activator of adenylate cyclase [30], and PMA, an activator of protein kinase C (Fig. 2A). When stimulated by 500 ng/mL of forskolin, melanin contents increased markedly. Forskolin did not show toxicity at concentrations up to 500 ng/mL, whereas PMA decreased cell viability at > 50 ng/mL (Fig. 2B). On the basis of these data, subsequent experiments were carried out using 500 ng/mL of forskolin as a stimulant. The time course of intracellular melanin production in HMV-II cells is shown in Fig. 2D. Intracellular production of melanin...
increased appreciably 48 h after the challenge with 500 ng/mL forskolin, reached a peak at 96 h and decreased to the level seen in controls within 120 h. Thus, melanin contents were determined 96 h after forskolin stimulation in subsequent experiments.

Next, melanin contents in various cell concentrations were measured. Maximal synthesis of melanin was observed at a density of $2 \times 10^5$ cells/mL (stimulated by 500 ng/mL forskolin) (Fig. 2 C). At a density of $2 \times 10^5$ cells/mL, cell viability and melanin content decreased (data not shown). Hence, subsequent experiments were carried out using a density of $2 \times 10^5$ cells/mL.

The effects of arbutin and kojic acid [31] as positive control agents to inhibit melanin synthesis were assessed. Arbutin (Fig. 3 A) and kojic acid (Fig. 3 B) appreciably reduced the melanin synthesis induced by forskolin at the indicated time points. These inhibitions were concentration-dependent (Fig. 3 C). The whitening effect of arbutin and kojic acid could be visualized under a microscope (Fig. 3 D).

Thus, investigation of the inhibitory effect of C. sappan was carried out using the in vitro assay system under the conditions mentioned above. The MeOH extract inhibited melanin synthesis by 84.5% at a 10 µg/mL concentration (Table 2). Furthermore, most of the activity was transferred into EtOAc and n-BuOH fractions. The H2O fraction also inhibited melanin synthesis but showed marked cytotoxicity.

To identify the active constituent(s), the EtOAc fraction was further separated using SiO2 or ODS column chromatography and HPLC. A new brazilein derivative (1) was isolated along with six known compounds (2–7) identified as sappanchalcone (2), 3′-deoxy-4-O- methylsappanol (3), brazilein (4), brazilein (5), sappanol (6), and 4-O-methylsappanol (7) by analyses of their NMR data and comparison of their spectroscopic data with those in the literature [20–24]. All six known compounds (2–7) were reportedly isolated from sappanwood [20–24]. Compound 1 showed a quasimolecular ion peak at m/z 285 [M – H]+. The molecular formula was determined to be C16H13O5 on the basis of HRFABMS and was identical to that of brazilein (5). The NMR data of 1 (Table 1) were similar with those of brazilein (5) [22], except for two aromatic proton signals. Thus, aromatic proton signals due to ring D at δ 6.81 (1H, s, H-8) and δ 6.92 (1H, s, H-11) in brazilein (5) were observed as two doublets at δ 6.47 (1H, d, J = 8.2 Hz, H-8) and δ 6.62 (1H, d, J = 8.5 Hz, H-9) in 1, which indicated that the location of the OH group at C-9 in brazilein (5) was at C-11 in 1. This was supported by analyses of the heteronuclear multiple bond correlation (HMBC) (Table 1 and Fig. 4 A) and also by the correlated spectroscopy (COSY) spectrum (correlation from H-8: δ 6.47 to H-9: δ 6.62 was observed). A significant nuclear Overhauser effect (NOE) correlation (Fig. 4 B) was also observed between H-11b and the OH group (C-6a), indicating a cis relationship. Furthermore, the CD spectrum of 1 showed a negative Cotton effect at UV 282 nm (Fig. 4 C), similar to that of brazilein (5) (data not shown). Thus, compound 1 was determined to be (6aS,11bR)-7,11b-dihydro-6H-indeno[2,1-c]chromene-3,6a,10,11-tetrol.

After establishing the structures, the inhibitory activities of these compounds on melanin synthesis were investigated. Among the seven compounds, brazilein (5) and O-methylsappanol (7) strongly suppressed melanin synthesis in cultured HMV-II cells with concentration values that gave a half-maximal response (EC50) of 3.0 ± 0.5 and 4.6 ± 0.7 µM, respectively. The five compounds (2, 3, 4, 5, and 7) showed cytotoxic effects, but selectivity factors (IC50/EC50) ranging from 1.43 (for 3), 1.82 (for 4), 1.95 (for 2), 4.39 (for 7), to 6.13 (for 5) were observed (Table 3). At a lower concentration, 1 µM of brazilein (5) inhibited melanin synthesis by 53%, and the cell viability was 94%.

Interestingly, the inhibitory activity as judged by the half-maximal effective concentration (EC50; 61.0 ± 3.1 µM) of the new compound (1), an isomer of brazilein (5), was > 20-times weaker than...
through the experiment. The expression for three mRNAs encoding tyrosinase, TYRP1, and TYRP2/DCT was upregulated by adding 500 ng/mL forskolin for 8 h compared with that of the non-treated control group (Fig. 5A).

Arbutin and kojic acid (the positive control agents) reduced the gene expression for tyrosinase and TYRP1 induced by 50 µM forskolin but did not reduce the gene expression for TYRP2/DCT (Fig. 5). The MeOH extract at 10 µg/mL concentration reduced the gene expression for tyrosinase but did not influence the mRNAs for TYRP1 and TYRP2/DCT (Fig. 5). Brazilin (5) reduced the expression of mRNAs for tyrosinase and TYRP2/DCT at the 10 µM concentration (Fig. 5A, B, and D) and reduced the latter more effectively (Fig. 5B and D). The MeOH extract and brazilin (5) did not reduce the expression of TYRP1 mRNA.

Discussion

The human melanoma HMV-II cells synthesized intracellular melanin within 96 h in response to forskolin without causing cytotoxicity. PMA also triggered melanin synthesis [32], whereas melanogenesis was lower than that seen for forskolin, and its action was accompanied by cytotoxicity. This finding suggested that forskolin was a more suitable stimulator than PMA for triggering melanogenesis in this culture system. The well-known whitening agents arbutin and kojic acid [31] appreciably suppressed melanin synthesis in HMV-II cells, suggesting that this assay system can be used to investigate inhibitors of melanin synthesis.

By using this assay system, the MeOH extract of sappanwood was found to inhibit melanin synthesis, and six active compounds were isolated. The five tested compounds (2, 3, 4, 5, and 7) and positive control agent (kojic acid) showed cytotoxicity against HMV-II cells. However, significant anti-melanogenic activities (EC\textsubscript{50}) were observed when cells were treated with these samples at two- or four-times lower concentrations. Brazilin (5) exhibited strong suppression of melanogenesis (EC\textsubscript{50} = 3.0 ± 0.5 µM), and cell viability was ≥ 95% at this concentration. Furthermore, 3’deoxy-4-O-methylsappanol (7) also suppressed melanogenesis with an IC\textsubscript{50} of 4.6 ± 0.7 µM, whereas nonsignificant cytotoxicity (cell viability <92%) was shown at this concentration. Therefore, the anti-melanogenic activity of the tested compounds was prob-
ably not due to their cytotoxicities. We are currently investigating the cytotoxic activity of isolated compounds on cultured human skin melanocytes, keratinocytes, and fibroblast cells to clarify this issue.

In addition, compound 1, which is an analog of 5, moderately inhibited melanogenesis, suggesting that the position of the benzoyl hydroxyl group is important for inhibition.

To clarify the mechanism of inhibition of brazilin (5), semiquantitative RT-PCR analyses of tyrosinase-related mRNAs in HMV-II cells were carried out. Arbutin and kojic acid suppressed the expression of tyrosinase and TYRP1, but did not act upon TYRP1 mRNA. Lee et al. [33] reported that the flavanone butin inhibited melanin synthesis in human epidermal melanocytes and suppressed the expression of tyrosinase, TYRP1, and TYRP2/DCT mRNAs. Therefore, the mechanism of inhibition of brazilin (5) is different from those of arbutin, kojic acid, and butin.

In the present study, we revealed the suppressive effect of the MeOH extract of sappanwood upon melanin synthesis in cultured human melanoma HMV-II cells and isolated six active compounds, one of which, compound 7, was a new brazilin analog. We also demonstrated that 5, which showed the most potent inhibition, selectively suppressed the expression of mRNAs for tyrosinase and TYRP2. These results support the traditional use of sappanwood for treating skin damage or as a facial cleanser. These compounds are candidates for new cosmetic agents.

### Table 3 Inhibitory activities of isolated compounds on melanin synthesis in cultured human melanoma HMV-II cells costimulated with forskolin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀ value for anti-melanogenic activity (µM)</th>
<th>IC₅₀ Value for cytotoxicities (µM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61.0 ± 3.1</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>42.6 ± 1.8</td>
<td>83.1 ± 4.0</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>50.4 ± 2.0</td>
<td>72.0 ± 2.4</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>18.6 ± 1.1</td>
<td>33.8 ± 1.1</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>3.0 ± 0.5</td>
<td>18.4 ± 0.8</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 100</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>4.6 ± 0.7</td>
<td>20.2 ± 0.8</td>
<td>8</td>
</tr>
<tr>
<td>Arbutin</td>
<td>123.3 ± 3.9</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>70.6 ± 3.0</td>
<td>99.7 ± 2.1</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are mean values from two independent experiments (n = 4/experiment). Cell viabilities were measured using the MTT method described in Materials and Methods, and IC₅₀ values for individual compounds against HMV-II cells were calculated. ND: not determined.

---

**Fig. 5** Effects of the MeOH extract, brazilin (5), arbutin, and kojic acid on the expression of mRNAs related to melanin synthesis in cultured HMV-II cells. Cells were treated with test samples [kojic acid, 100 µM; arbutin, 100 µM; brazilin (5), 5 µM; MeOH extract, 10 µg/mL]. mRNAs were extracted 8 h after incubation, and the expression of tyrosinase (TYR), TYRP1, or TYRP2/DCT was measured by semiquantitative RT-PCR using specific primers (panel A). β-Actin gene expression (ACT) was used as an endogenous internal standard. Mean expression values for non-treated control cultures are normalized to the expression of ACT. Expression levels of TYR (panel B), TYRP1 (panel C), and TYRP2/DCT (panel D) are indicated as the means ± SD of three independent experiments.
Conflict of Interest

The authors declare no conflict of interest.

References

13 Hulme AN, McNab H, Peggie DA, Quye A. Negative ion electrospray mass spectrometry of neoflavonoids. Phytochemistry 2005; 66: 2766–2770