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Extracellular melanogenesis inhibitory activity and the structure-activity relationships of ugonins from *Helminthostachys zeylanica* roots

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ABSTRACT

Ugonin J, K, and L, which are luteolin derivatives, were isolated from *Helminthostachys zeylanica* roots by a series of chromatographic separations of a 50% ethanol/water extract. They were identified using nuclear magnetic resonance (NMR), ultraviolet (UV) spectra, and ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC–TOF-MS). In this study, the intra and extracellular melanogenic activity of the ugonins were determined using B16 melanoma cells. The results showed that ugonin J at 12.5, 25, and 50 μM reduced extracellular melanin contents to 75, 16, and 14%, respectively, compared to the control. This indicates that ugonin J showed a stronger activity than arbutin, used as the positive control. Moreover, ugonin K showed a more potent inhibition with 19, 8, and 9% extracellular melanin reduction at the same concentrations, than that shown by ugonin J. In contrast, ugonin L did not inhibit intra- or extracellular melanogenic activity. Furthermore, in order to investigate the structure–activity relationships of the ugonins, the intra- and extracellular melanogenic activity of luteolin, methylluteolin, quercetin, eriodictyol, apigenin, and chrysin were determined. Consequently, it was suggested that the catechol and flavone skeleton of ugonin K is essential for the extracellular melanogenic inhibitory activity, and the low polarity substituent groups on the A ring of ugonin K may increase the activity.

1. Introduction

Melanin is a pigment found in the skin and is thought to play an important role in protecting the skin from ultraviolet (UV) rays, thereby preventing skin cancer [1,2]. However, excessive accumulation of melanin in the skin surface results in mottled skin; mottles are unfavorable to esthetics and health. Melanin is biosynthesized in melanocyte cells from L-tyrosine. The key enzyme involved in melanin biosynthesis is tyrosinase, which contains copper and catalyzes two reactions in this process [3]. In the cells, the first key step in melanin biosynthesis is the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and the second is the oxidation of L-DOPA to L-DOPA-quinone by the enzymatic reaction of tyrosinase. This results in the production of the red-orange-colored pheomelanin and blackish-brown-colored eumelanin [3]. The melanin biosynthesized in melanocytes is transported to the keratinocytes present on the skin surface. Skin pigmentation subsequently occurs by the cornification of keratinocytes and then, the mottles form from the melanin released outside of the melanocytes. Intracellular melanin status is usually determined using B16 melanoma cells to screen compounds, which elicit melanogenic controlling activity [4–6]. Extracellular melanin was the focus of this study. Therefore, the levels of both intra- and extracellular melanin were determined using B16 melanoma cells to clarify the importance of the extracellular melanin in the development of skin whitening agents. Furthermore, in this study, the melanogenic activity of components of the *Helminthostachys zeylanica* root extract was investigated by determining the absorbance of intra- and extracellular melanin following extract treatment.

H. zeylanica, belongs to the Ophioglossaceae family, and is used as a traditional folk medicine in the treatment of ailments as an antipyretic, antiphlogistic, anodyne [7], antimalarial agent, and for the treatment of sciatica and ulcer boils [8]. Previous studies have isolated ugonins A–T from *H. zeylanica* extracts and investigated their antioxidant and anti-inflammatory activity [9–11]. Furthermore, a novel quercetin glycoside, which stimulated intercellular melanogenesis, was isolated and identified from the *H. zeylanica* root extracts [12]. In this study, we further investigated the intra- and extracellular melanogenic activity of the extract components. Moreover, this is the first study to report the importance of determining extracellular melanogenesis in the search for useful ingredients for whitening agents. This was achieved by

Abbreviations: ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; L-DOPA, L-3,4-dihydroxyphenylalanine; MITF, microphthalmia-associated transcription factor; MAPK, mitogen-activated protein kinase.

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determining the melanogenic inhibitory effect and structure–activity relationship of the ugonins isolated from the *H. zeylanica* root extracts. In addition, we determined the levels of intra- and extracellular melanin.

2. Materials and methods

2.1. General experimental procedures

The proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded in methanol-d_4 using a Jeol EC 600M Hz NMR spectrometer. The coupling constants were expressed in Hz, and the chemical shifts were determined on a δ (ppm) scale. The ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC–TOF-MS, Waters^R Xevo^{TM} TQ mass spectrometer) was performed using a C_{18} column (2.1 mm $\varphi \times$ 100 mm L, Waters, Milford, MA). The UPLC–TOF-MS data were collected in negative ionization modes. The capillary voltage was 3.0 kV. The cone and desolvation gas flow rates were set at 50 and 1000 L/h, respectively, and the source and desolvation temperatures were 150 and 500 °C, respectively. The UV spectra were recorded on a Shimadzu SPD-M20A diode array detector. The preparative high-performance liquid chromatography (HPLC, Jasco) was performed using an Inertsil ODS-3 column (10 mm $\varphi \times$ 250 mm L, GL Sciences, Tokyo, Japan). Synthetic melanin was purchased from Sigma-Aldrich (St. Louis, MO). The other products were commercially available and purchased from Wako Chemicals (Richmond, VA).

2.2. Materials

The *H. zeylanica* root powder was collected from Samarinda, Indonesia in 2006. The sample and voucher specimen (number WAN0017365) were deposited at the Herbarium at Wanariset, Samboja, East Kalimantan, Indonesia.

2.3. Isolation and identification of ugonins from H. zeylanica root powder extract

The *H. zeylanica* root powder (about 100 g) was extracted with a 50% ethanol and water mixture. The extract was separated using Sephadex LH20 gel column (25 mm $\varphi \times$ 820 mm L) chromatography and eluting with a solvent mix of water:methanol (MeOH, 1:1 v/v), to obtain fractions LHFr.1–6. The LHFr.6 fraction was further separated using the same chromatographic procedure except that the eluting solvent was a mixture of water:MeOH at a ratio of 2:3 (v/v) and subfractions LHFr.6A–6G were obtained. Ugonin J, K, and L were isolated from subfractions LHFr.6E, 6F, and 6G respectively, using preparative HPLC (Inertsil® ODS-3 V, 10 mm $\varphi \times$ 250 mm L, isocratic elution with MeOH:0.05% aqueous trifluoroacetic acid, TFAaq., 95:5).

The isolated ugonins were identified using ^{1}H NMR, ^{13}C NMR, and UV spectral analysis, as well as UPLC–TOF-MS. The UPLC–TOFMS measurements were performed using a C_{18} (2.1 × 100 mm) column with MeOH:water as the eluent with the following schedule: 5:95 (0 min), 100:0 (10 min), 100:0 (13 min). The physical descriptions and spectral data are as follows:

Ugonin J: yellow powder; UV λ_{max} 214, 274, 345 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 0.91 (3H, s, H-13), 1.03 (3H, s, H-12), 1.22 (1H, m, H-14), 1.47 (1H, m, H-15), 1.57 (1H, m, H-15), 1.66 (1H, m, H-14), 1.92 (1H, m, H-16), 2.37 (1H, dd, J = 12.1, 4.08 Hz, H-10), 2.50 (1H, m, H-16), 2.68 (1H, dd, J = 13.2, 4.07 Hz, H-9), 2.91 (1H, t, J = 11.7 Hz, H-9), 4.17 (1H, br s, H-18), 4.39 (1H, d, J = 2.04 Hz, H-18), 6.37 (1H, s, H-8), 6.47 (1H, s, H-3), 6.86 (1H, d, J = 6.90 Hz, H-5′), 7.31 (1H, dd, J = 6.90, 2.04 Hz, H-6′), 7.32 (1H, d, J = 2.04 Hz, H-2′); ^{13}C NMR (CD_3OD,150 MHz) δ_C 21.0 (C-9), 23.2 (C-15), 27.1 (C-13), 27.3 (C-12), 30.9 (C-16), 34.1 (C-14), 34.5 (C-11), 52.1 (C-10), 92.6 (C-8), 102.4 (C-3), 103.6 (C-4a), 108.3 (C-18), 112.1 (C-6), 112.8 (C-2′), 115.4 (C-5′), 118.8 (C-6′), 122.5 (C-1′), 145.8 (C-3′), 149.3 (C-4′), 149.7 (C-17), 155.7 (C-8a), 159.0 (C-5), 162.8 (C-7), 164.5 (C-2), 182.6 (C-4); UPLC–TOFMS m/z 421.165 $[M-H]^-$.

Ugonin K: yellow powder; UV λ_{max} 215, 275, 344 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 0.91 (3H, s, H-13), 1.04 (3H, s, H-12), 1.23 (1H, m, H-14), 1.49 (1H, m, H-15), 1.58 (1H, m, H-15), 1.59 (1H, m, H-14), 1.94 (1H, m, H-16), 2.25 (1H, dd, J = 11.7, 4.14 Hz, H-10), 2.47 (1H, m, H-16), 2.72 (1H, dd, J = 13.1, 4.14 Hz, H-9), 2.91 (1H, t, J = 12.4 Hz, H-9), 3.88 (3H, s, 7OCH_3), 4.08 (1H, d, J = 1.20 Hz, H-18), 4.37 (1H, d, J = 2.04 Hz, H-18), 6.54 (1H, s, H-3), 6.61 (1H, s, H-8), 6.88 (1H, d, J = 8.22 Hz, H-5′), 7.36 (1H, d, J = 2.76 Hz, H-2′), 7.39 (1H, dd, J = 8.28, 2.10 Hz, H-6′); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 20.8 (C-9), 23.0 (C-15), 27.3 (C-12, 13), 30.7 (C-16), 34.1 (C-14), 34.2 (C-11), 52.8 (C-10), 55.0 (7OCH_3), 89.3 (C-8), 102.8 (C-3), 104.6 (C-4a), 108.3 (C-18), 112.7 (C-6), 112.8 (C-2′), 115.4 (C-5′), 119.0 (C-6′), 122.6 (C-1′), 143.3 (C-3′), 145.7 (C-4′), 149.7 (C-17), 155.3 (C-8a), 158.0 (C-5), 163.4 (C-7), 164.1 (C-2), 182.7 (C-4); UPLC–TOFMS m/z 435.186 $[M-H]^-$.

Ugonin L: yellow powder; UV λ_{max} 215, 271, 336 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 0.94 (3H, s, H-13), 1.02 (3H, s, H-12), 1.37 (1H, dd, J = 14.4, 6.18 Hz, H-14), 1.50 (1H, br d, J = 13.1 Hz, H-14), 1.63 (1H, dd, J = 13.1, 4.80 Hz, H-10), 1.70 (3H, m, H-15,16), 2.11 (1H, br d, J = 12.4 Hz, H-16), 2.32 (1H, dd, J = 16.5, 13.0 Hz, H-9), 2.73 (1H, dd, J = 17.2, 4.80 Hz, H-9), 3.96 (3H, s, 7OCH_3), 6.48 (1H, s, H-3), 6.70 (1H, s, H-8), 6.88 (1H, d, J = 8.22 Hz, H-5′), 7.34 (1H, d, J = 2.10 Hz, H-2′), 7.36 (1H, dd, J = 8.22, 2.04 Hz, H-6′); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 17.8 (C-9), 18.4 (C-12), 19.5 (C-18), 19.6 (C-14), 31.1 (C-11), 39.1 (C-13), 41.2 (C-16), 46.7 (C-10), 55.3 (7OCH_3), 78.8 (C-17), 90.4 (C-8), 105.5 (C-3), 108.0 (C-4a), 108.7 (C-6), 112.6 (C-2′), 115.6 (C-5′), 118.5 (C-6′), 124.2 (C-1′), 143.8 (C-3′), 149.1 (C-4′), 153.6 (C-5), 158.2 (C-8a), 162.4 (C-7), 162.5 (C-2), 185.7 (C-4); UPLC–TOFMS m/z 435.185 $[M-H]^-$.

2.4. Tyrosinase activity assay

The tyrosinase activity measurements were performed using a previously described method [13,14]. The sample (60 μL) was placed in a 96-well plate and 30 μL of mushroom tyrosinase (333 U/mL in phosphate buffer, 50 mM, pH 6.5) and 110 μL of substrates (2 mM L-tyrosine or 2 mM L-DOPA) were added. After incubation at 37 °C for 30 min, the absorbance was measured at 510 nm using a microplate reader. The tyrosinase activity was expressed as the half-maximal inhibitory concentration (IC_{50}), which is the concentration of the samples producing 50% inhibition.

2.5. Cell culture

The mouse melanoma B16-F0 cells (DS Pharma Biomedical, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.6. Measurement of cellular melanin content

The measurement of cellular melanin was performed according to a previously described method [15]. In brief, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and detached using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA). The cells were placed in a 24-well plate (5.0×10^4 cells/well) and allowed to adhere at 37 °C for 24 h. The samples were added to the cells followed by incubation for 72 h, and then 200 μL of the culture medium was added to a 96-well plate. The extracellular melanin content was determined by measuring the absorbance of the medium at 510 nm using a microplate reader. The cells were then washed with PBS followed by lysis in 600 μL of 1 M sodium hydroxide (NaOH) with heating at 100 °C for 30 min to solubilize the melanin. The resulting lysate (250 μL) was placed in a 96-well microplate, and the intracellular melanin contents were determined by measuring the absorbance at 405 nm using a microplate reader. Each experiment was repeated

twice. The melanin production activity was expressed as a percentage of that of the control cells treated with dimethyl sulfoxide (DMSO) alone.

2.7. Cell viability

The cell viability measurement was performed according to a previously described method [16]. The cell viability was determined using the microculture tetrazolium technique (MTT). Cell cultures were initiated in 24-well plates at a density of 5.0×10^4 cells/well. After incubation, 50 μL of the MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide in PBS, 5 mg/mL) was added to each well. The plates were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 4 h. After the medium was removed, 1.0 mL isopropyl alcohol (containing 0.04 N hydrochloric acid, HCl) was added to the plate, and 150 μL was added to a 96-well plate. The absorbance was measured at 590 nm using a microplate reader. Each experiment was repeated twice. The cell viability was expressed as a percentage of that of the control cells treated with the DMSO vehicle alone.

2.8. Quantitative determination of the intra- and extracellular melanin

The intra- and extracellular melanin content of the control cells treated with the DMSO vehicle alone was determined using the standard curve method. Similar to the procedure for determining the intracellular melanin content, the standard synthesized melanin was dissolved in 1 N NaOH and heated for 30 min at 100 °C. Then, 250 μL of melanin solution was placed in a 96-well microplate, and the absorbance was measured at 405 nm with a microplate reader to obtain the standard curve. To determine the extracellular melanin quantity, the standard synthesized melanin was dissolved in DMEM and 200 μL of the melanin solution was placed in a 96-well microplate, and the absorbance was measured at 510 nm with a microplate reader to obtain a standard curve to quantify the extracellular melanin contents.

2.9. Synthesis of methylluteolins

2.9.1. Synthesis of 7,4′-O-dimethylluteolin

Luteolin (100 mg, 0.350 mmol), potassium carbonate (K_2CO_3, 193 mg, 1.40 mmol), and dimethyl sulfate (133 μL, 1.40 mmol) were added to 10 mL of dimethylformamide (DMF) and the mixture was stirred for 3 h at 60–65 °C. The resulting mixture was then diluted with 50 mL of ethyl acetate (EtOAc) and washed with water (2 × 50 mL). The EtOAc phase was dried using sodium sulfate (Na_2SO_4), and the reactant was obtained by evaporating the solvent. Then 30 mL of MeOH were added to the reactant, and the precipitate was obtained as 7,4′-*O*-dimethylluteolin with a yield of 20.9% by filtration. The structure of the synthesized 7,4′-*O*-dimethylluteolin was confirmed using NMR and UV spectra as well as UPLC–TOF-MS.

2.9.2. Synthesis of 7-O-methylluteolin and 4′-O-methylluteolin

Luteolin (100 mg, 0.350 mmol), K_2CO_3 (48.3 mg, 0.350 mmol), and dimethyl sulfate (33.3 μL, 0.350 mmol) were added to 10 mL of DMF and the mixture was then stirred for 3 h at 60–65 °C. The resulting mixture was diluted with 50 mL of EtOAc and washed with water (2 × 50 mL). The EtOAc phase was dried using Na_2SO_4, and the reactant was obtained by evaporating the solvent. The 7-*O*-methylluteolin and 4′-*O*-methylluteolin obtained from luteolin were yellow powders with yields of 10.0 and 20.2% respectively. They were purified using preparative HPLC with an ODS-3 column (10 mm φ × 250 mm L) eluted using a linear gradient of MeOH:0.05% TFA aq., and the following schedule: 60:40 (0 min), 100:0 (50 min), and 100:0 (60 min). The structures of the synthesized 7-*O*-methylluteolin and 4′-*O*-methylluteolin were confirmed using NMR and UV spectral analysis, as well as UPLC–TOF-MS. The physical descriptions and spectral data are as follows:

7,4′-*O*-dimethylluteolin: yellow powder; UV λ_{max} 216, 262, 350 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 3.83 (6H, s, 4′OCH_3, 7OCH_3), 6.34 (1H, br s, H-6), 6.70 (1H, br s, H-8), 6.77 (1H, s, H-3), 7.06 (1H, d, J = 8.2 Hz, H-5′), 7.42 (1H, br s, H-2′), 7.53 (1H, br s, J = 8.9 Hz, H-6′); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 56.3 (7OCH_3), 56.6 (4′OCH_3), 93.2 (C-8), 98.5 (C-6), 104.2 (C-3), 105.2 (C-4a), 112.6 (C-5′), 113.6 (C-2′), 119.4 (C-6′), 123.4 (C-1′), 147.3 (C-3′), 151.8 (C-4′), 157.8 (C-8a), 161.7 (C-5), 164.4 (C-2), 165.7 (C-7), 182.4 (C-4); UPLC–TOFMS m/z 313.058 $[M - H]^-$.

4′-*O*-methylluteolin: yellow powder; UV λ_{max} 206, 252, 346 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 3.92 (3H, s, 4′OCH_3), 6.23 (1H, br s, H-6), 6.53 (1H, br s, H-8), 6.62 (1H, s, H-3), 7.10 (1H, d, J = 8.2 Hz, H-5′), 7.47 (1H, d, J = 2.1 Hz, H-2′), 7.53 (1H, dd, J = 8.2, 2.0 Hz, H-6′); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 55.6 (4′OCH_3), 93.9 (C-8), 98.9 (C-6), 103.9 (C-3), 104.5 (C-4a), 111.6 (C-5′), 112.8 (C-2′), 118.9 (C-6′), 124.0 (C-1′), 147.0 (C-3′), 150.9 (C-4′), 158.0 (C-8a), 162.5 (C-5), 164.0 (C-2), 164.1 (C-7), 182.4 (C-4); UPLC–TOFMS m/z 299.053 $[M - H]^-$.

7-*O*-methylluteolin: yellow powder; UV λ_{max} 207, 254, 349 nm; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 3.91 (3H, s, 7OCH_3), 6.29 (1H, br s, H-6), 6.60 (1H, br s, H-3), 6.66 (1H, s, H-8), 6.99 (1H, d, J = 8.2 Hz, H-5′), 7.46 (1H, br d, J = 9.3 Hz, H-6′), 7.52 (1H, br s, H-2′); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 55.6 (7OCH_3), 92.4 (C-8), 97.8 (C-6), 103.4 (C-3), 105.1 (C-4a), 113.5 (C-2′), 115.9 (C-5′), 119.3 (C-6′), 122.7 (C-1′), 145.7 (C-3′), 149.4 (C-4′), 157.6 (C-8a), 157.9 (C-2), 162.2 (C-5), 165.7 (C-7), 182.3 (C-4); UPLC–TOFMS m/z 299.052 $[M - H]^-$.

3. Results and discussion

3.1. Identification of compounds

The isolated ugonin J, K, and L were identified by comparing the data obtained in this study to previous results obtained by Huang et al. (2003) [10]. The chemical shifts of the B and C rings observed in the ^{1}H NMR spectra were almost similar for all three compounds. In contrast, three singlet protons were observed around 3.9 ppm in the spectra of ugonin K and L, which were not observed in the ^{1}H NMR spectra of ugonin J. This suggests the existence of a methoxyl group in the structures of ugonin K and L, which was further confirmed by the hetero-nuclear multiple-bond correlation (HMBC) data (not shown). The differences between ugonin K and L were shown by the differences in the chemical shift of C-17 and C-18, which were observed at 149.7 and 108.3 ppm, respectively for ugonin K, and 78.8 and 19.5 ppm, respectively, for ugonin L. This result indicated that the C-17 and C-18 of ugonin L were not linked by a double bond. Moreover, the chemical shift of C-17 corroborated the ring structure of ugonin L (Fig. 1). The UPLC–TOF-MS data also corroborated each structure.

The synthesized methylluteolins were also identified using the same analytical instruments, and the position of their methoxyl groups were clarified using HMBC. As shown in Fig. 2, the protons of the methoxyl group in 7-*O*-methylluteolin correlated with the carbon binding to the methoxyl group. The location of this group was identified as position 7 on the luteolin structure, using the other key HMBC correlations between the H-6 and C-7, or H-8 and C-7. Similarly, the HMBC correlations were used to clarify the exact position of the methoxyl groups in the other synthesized methylluteolins.

3.2. Inhibition of melanogenesis by ugonins and their tyrosinase activity

The quantitative determination of the intra- and extracellular melanin of the B16 melanoma cells revealed that the control levels were 6.2 and 140 μg, respectively, per well. This result shows that the level of the extracellular melanin is approximately 23-fold higher than that of the intracellular melanin, which provided us with convincing evidence to develop the whitening agent. There are numerous reports that have only determined intracellular melanin in the process of investigating compounds that are suspected to regulate melanogenesis.

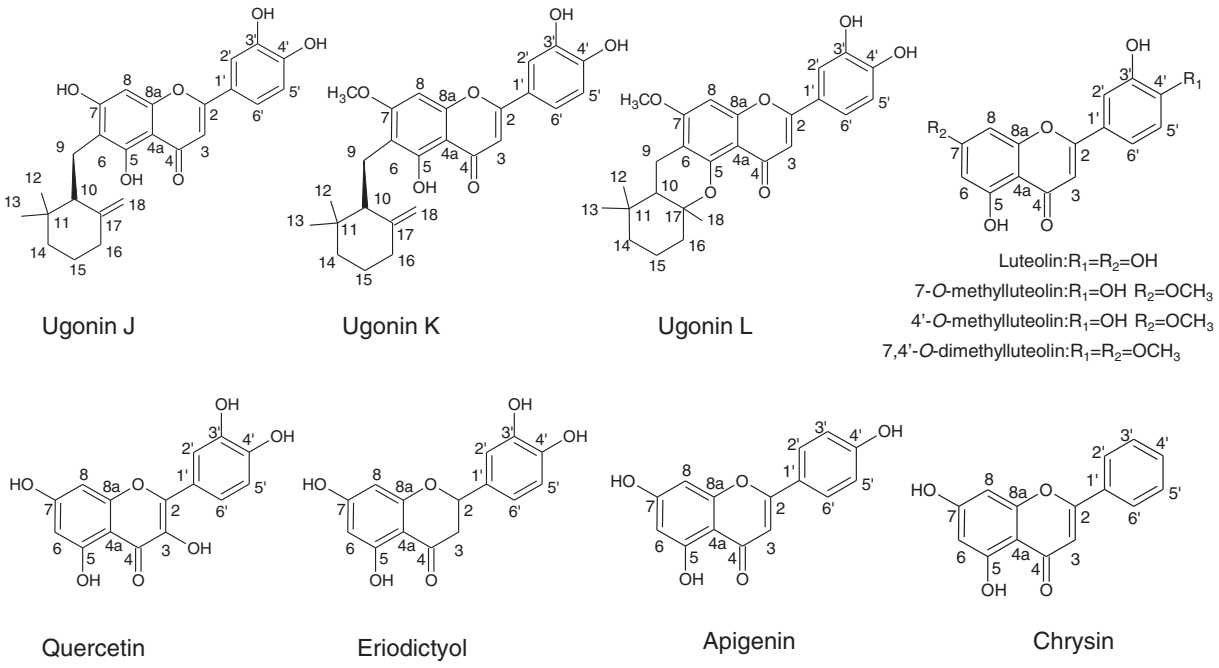

Fig. 1. Structures of ugonins and compounds used for investigation of structure–activity relationships.

However, the result of our quantitative determination shows that most of the melanin synthesized by the B16 melanoma cells was released outside the cells. Therefore, to elucidate the regulation of melanogenesis by the compounds under investigation, it is essential to determine not only the intracellular but also the extracellular activity of the compounds.

The results of both the intra- and extracellular melanogenic activity of the ugonins isolated from the 50% ethanol/water extract of the roots of *H. zeylanica* are shown in Table 1. The results revealed that the extracellular melanogenic activities of ugonin J at 12.5, 25, and 50 μM were 75, 16, and 14%, respectively, compared to the control, which had 100% melanogenic activity. This result suggests that the effect of ugonin J was stronger than that of arbutin, which was used as the positive control. Moreover, the inhibitory activity of ugonin K was more potent than that shown by ugonin J, with 19, 8, and 9% extracellular melanogenic activity at the same concentrations. Interestingly, ugonin J and K showed no inhibition of intracellular melanogenesis, suggesting that they may inhibit the transportation or release of melanosome or both, in B16 melanoma cells as well as inhibit melanin biosynthesis. In contrast, ugonin L did not show intra- or extracellular inhibition of melanogenesis. Comparing the melanogenic activity of ugonin J, K, and L, the C-6 substituent cyclogeranyl group of ugonin J and K may play an important role in their inhibitory effects. This is because the inhibitory activity appears to have been abrogated by the formation of a ring with the 5-hydroxyl group as was observed with the structure and lack of activity of ugonin L. Furthermore, the presence of the 7-methoxyl group in ugonin K may increase its inhibitory effect on extracellular melanogenesis.

To further investigate the importance of the C-6 substituent cyclogeranyl and the 7-methoxyl group for the inhibition of extracellular melanin biosynthesis, luteolin and the synthesized 7-*O*-methylluteolin were used as targets for comparison. Consequently, the inhibition of extracellular melanogenesis by luteolin was lower than that exerted by 7-*O*-methylluteolin, ugonin J, and K at 25 and 12.5 μM. In addition, luteolin and 7-*O*-methylluteolin showed higher cytotoxicity than ugonin J and K. These results confirm that the 7-methoxyl and C-6 substituent cyclogeranyl groups of ugonin K are important for the inhibitory activity against extracellular melanin biosynthesis. Additionally, it was shown that the substituent groups that bind to the A ring of ugonin J and K may increase the cell viability.

Next, to clarify the role of the B and C rings of ugonin J and K in their structure–activity relationships, the melanogenic activities of the commercially available quercetin, eriodictyol, apigenin, and chrysin and the synthesized 4′-*O*-methylluteolin and 7,4′-*O*-dimethylluteolin were determined. Quercetin is known as the intracellular melanogenesis inhibitor [17]. This study also shows that quercetin reduces the intracellular melanogenesis with 56% at 50 μM. However, quercetin and eriodictyol, which have substituents on the C ring that differ from

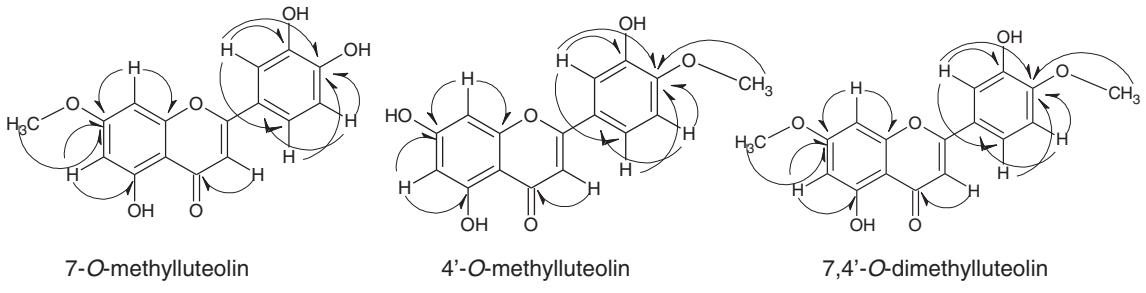

Fig. 2. Key HMBC correlations of synthesized methylluteolins.

Table 1
Intra- and extracellular melanogenesis activities and cell viabilities of ugonins and compounds used for investigation of structure–activity relationships.

Compound name and concentration (μM)	Cell viability (%)	Intracellular melanogenesis activity (%)	Extracellular melanogenesis activity (%)
Ugonin J			
50.0	62 ± 5.4**	106 ± 13.6	14 ± 1.6**
25.0	100 ± 1.0	88 ± 7.7	16 ± 1.6**
12.5	105 ± 0.6	99 ± 5.9	75 ± 36.7
Ugonin K			
50.0	79 ± 15.5	93 ± 4.1	9 ± 2.0**
25.0	95 ± 7.1	107 ± 10.7	8 ± 0.8**
12.5	110 ± 1.9	122 ± 18.0	19 ± 2.0**
Ugonin L			
50.0	111 ± 0.1	110 ± 0.4	81 ± 9.6
25.0	114 ± 0.1	96 ± 6.3	78 ± 5.3
12.5	106 ± 0.0	113 ± 1.8	92 ± 2.7
Luteolin			
50.0	69 ± 0.6	52 ± 3.7	13 ± 0.1**
25.0	75 ± 3.5	60 ± 5.1	70 ± 11.2
12.5	88 ± 0.1	90 ± 7.5	132 ± 3.5
7-*O*-methylluteolin			
50.0	80 ± 5.8	56 ± 10.5	15 ± 0.0**
25.0	64 ± 8.6*	103 ± 2.6	22 ± 2.7*
12.5	70 ± 0.9*	118 ± 1.5	57 ± 0.4
4′-*O*-methylluteolin			
50.0	88 ± 1.7	124 ± 15	78 ± 1.1
25.0	104 ± 8.8	100 ± 8.5	78 ± 3.0
12.5	105 ± 6.7	102 ± 7.4	78 ± 10.1
7,4′-*O*-dimethylluteolin			
50.0	97 ± 1.3	114 ± 16.2	101 ± 7.1
25.0	93 ± 0.0	107 ± 2.7	105 ± 7.8
12.5	90 ± 5.4	115 ± 10.6	110 ± 9.6
Quercetin			
50.0	94 ± 1.7	56 ± 1.8	104 ± 7.0
25.0	105 ± 1.0	84 ± 3.5	109 ± 1.8
12.5	110 ± 1.3	94 ± 7.0	99 ± 2.1
Eriodictyol			
50.0	71 ± 1.3**	193 ± 73.4	135 ± 11.8
25.0	93 ± 7.2	117 ± 2.3	106 ± 5.2
12.5	92 ± 3.5	124 ± 3.4	112 ± 8.6
Apigenin			
50.0	48 ± 1.8**	515 ± 94.2*	215 ± 4.6**
25.0	70 ± 1.1**	157 ± 6.1**	153 ± 0.5**
12.5	94 ± 2.7	106 ± 2.6	108 ± 5.1
Chrysin			
50.0	94 ± 1.7	100 ± 0.2	127 ± 1.2
25.0	105 ± 1.0	77 ± 3.1	116 ± 0.1
12.5	110 ± 1.3	72 ± 0.3	114 ± 0.3
Arbutin			
730.0	105 ± 0.7	86 ± 7.2	52 ± 12.7*

All data were expressed as the mean ± S.D. Differences were examined for statistical significance using Student's *t*-test. n = 2.
* $p < 0.05$ compared with respective control values.
** $p < 0.01$ compared with respective control values.

that of luteolin, showed no extracellular melanogenesis inhibitory activity. In addition, apigenin, chrysin, 4′-*O*-methylluteolin, and 7,4′-*O*-dimethylluteolin, which have substituents on the B ring that differ from those of luteolin or 7-*O*-methylluteolin, also showed no inhibitory activity. Furthermore, luteolin and its 7-*O*-methylate suppressed the extracellular melanin levels at 25 and 50 μM. Conversely, apigenin stimulated intra- and extracellular melanin biosynthesis. These results suggest that the flavone skeleton on the C and catechol skeleton on B rings of ugonin J, K, luteolin, and 7-*O*-methylluteolin may play a fundamental role in their inhibition of extracellular melanognesis.

The IC_{50} values of the tyrosinase activity of ugonin J, K, L, and the target agents with which they were compared are shown in Table 2. Ugonin J, K, luteolin, and 7-*O*-methylluteolin, which inhibited extracellular melanogenesis, did not inhibit tyrosinase. Therefore, these compounds may suppress the expression of tyrosinase, which is transcriptionally regulated by the microphthalmia-associated transcription factor (MITF). Furthermore, MITF expression is activated or suppressed by kinases such as the p38 mitogen-activated protein kinase (MAPK) cascade, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinase (JNK) [18]. Some melanogenic regulating agents were discovered by determining their effects on the expression of tyrosinase, p38, JNK, ERK, and MITF as well as tyrosinase activity [19–21]. In particular, the determination of the downregulation of the expression of activated p38 and MITF is commonly used to investigate the mechanism by which compounds inhibit melanogenesis [19,22]. In the case of ugonin K, it is noteworthy that previous studies have reported that p38 MAPK stimulation also plays an important role in osteoblastic differentiation [23]. In addition, studies suggest that the osteoblastic differentiation was promoted by ugonin K, which accelerates p38 MAPK in mouse MC3T3-E1 cells [24]. This study reported that ugonin K stimulates p38 MAPK, which upregulates the expression of tyrosinase. This result may be contrary to the results showing the inhibition of melanin biosynthesis by ugonin K in this study. Therefore, regarding the expression of tyrosinase, ugonin K may suppress extracellular melanin levels by a cascade other than that involving p38 MAPK. In addition, ugonin K may be involved in the transportation of melanosome because it reduced the extracellular melanin levels despite its lack of inhibitory effects on intracellular melanogenesis. Melanosome is transported by a wide variety of proteins in melanocyte such as actin, myosin Va, Rab27A, Slac2-a, and others. The mature melanosomes interact with myosin Va, which is a motor protein, via Rab27A localized on the mature melanosomes and Slp2-a [25,26]. The complex is transported by actin to the periphery of the cell from the perinuclear region of melanocytes [27]. Ugonin J and K may inhibit the transportation of the melanosomes by suppressing the expression or the activity of these proteins or both.

4. Conclusions

In this study, ugonin J, K, and L were isolated from *H. zeylanica* roots and identified using NMR and UV spectral analysis, as well as UPLC–TOF-MS. Ugonin J and K showed high inhibition of extracellular melanogenesis. The result of the structure–activity relationship investigation revealed that the flavone and catechol skeleton of ugonin J, K may be critical for its activity. In addition, the substituent groups that bind to

Table 2
Tyrosinase inhibitory activities of ugonins and compounds used for investigation of structure-activity relationships.

Substrates	L-Tyrosine	L-DOPA
	IC_{50} (μg/ml)	IC_{50} (μg/ml)
Ugonin J	>100	>100
Ugonin K	>100	>100
Ugonin L	>100	>100
Luteolin	>100	>100
7-*O*-methylluteolin	>100	>100
4′-*O*-methylluteolin	>100	>100
7,4′-*O*-dimethylluteolin	>100	>100
Quercetin	>100	34.8
Eriodictyol	>100	67.3
Apigenin	>100	>100
Chrysin	>100	>100
Kojic acid	21.5	0.030
Arbutin	>100	>100

the A ring of ugonin J and K may play an important role in increasing its activity. The level of extracellular melanin was considerably higher than that of intracellular melanin. In addition, skin pigmentation is caused by the melanin released outside from the melanocytes. Therefore, ugonin J and K may be useful lead compounds for the development of new whitening agents. Additional investigations are required to elucidate the mechanism of the activity of ugonin J and K. This may be achieved by determining the expression and activity of the proteins involved in the expression of tyrosinase or transportation of melanosomes.

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