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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Structure-activity relationship for vanilloid compounds from extract of *Zingiber officinale* var rubrum rhizomes: effect on extracellular melanogenesis inhibitory activity

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Abstract

The components in the methanol extract of *Zingiber officinale* var rubrum (red ginger) were isolated by a series of column chromatography and identified by Nuclear Magnetic Resonance (NMR) and Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOFMS). Thirteen components, including eleven vanilloid compounds, were isolated from the extract. All the isolated components reduced extracellular melanin contents. Structure-activity relationship studies suggested that elongation of the carbon chain of shogaol and gingerol increased the extracellular melanogenesis inhibitory activity, and that the carbonyl and hydroxyl groups on the carbon chain in gingerol played an important role in this effect. In order to reveal the importance of the vanillyl group for the melanogenesis inhibitory activity, 6-gingerol (**1**) and 6-shogaol (**5**), which were the main compounds in the extract, were glucosylated and their effects were evaluated. The glucosylated 6-shogaol resulted in improved melanogenesis inhibitory activity, while the glucosylated 6-gingerol had no such effect. These results indicated different mechanisms for the melanogenesis inhibitory activity of the two vanilloid compounds.

Keywords *Zingiber officinale* var rubrum · Vanilloid compounds · Melanogenesis · B16 melanoma

Introduction

Melanin protects the skin from the damage caused by ultraviolet light. However, excessive biosynthesis of melanin in melanocytes leads to hyperpigmentation and symptoms such as malaise, as well as freckles, melanoderma, and solar lentigo. In contrast, a decrease in melanin levels results in graying of hair and white spot disease. Melanogenesis is biosynthesized by enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2). Tyrosinase is a rate-limiting enzyme that catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and further oxidation to dopaquinone. (Fitzpatrick and Lerner 1949; Kameyama et al. 1995) The expression of melanogenic enzymes is stimulated by the microphthalmia-associated transcription factor (MITF), and the MITF expression is regulated by a signaling pathway such as the p38-mitogen activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) pathways. (Bertolotto et al. 1998; Busca et al. 2000; Bu et al. 2008; Jang et al. 2011; Lopez-Bergami 2011; Mitsunaga and Yamauchi 2015; Yamauchi and Mitsunaga 2016) Several medicinal plant extracts have been investigated as the source of candidates for melanogenesis regulating agents. Recently, pregnanes isolated from *Cynanchum atratum* have been demonstrated to show melanogenesis inhibitory activity in α-melanocyte stimulating hormone (α-MSH)-activated B16 melanoma cells. (Jin et al. 2018) Steroid compounds obtained from the extract of *Sauropus androgynus* L. Merr. leaves were found to inhibit melanogenesis by suppressing the expression of MITF, tyrosinase, TRP-1, and 2. (Zhang et al. 2018) On the other hand, melanogenesis

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stimulators have also been found in the extracts of medicinal plants. Punicalagin and α,β-terchebulin have been isolated and identified as melanogenesis stimulators from *Terminalia brownii* bark extract via a screening test using 104 plant extracts. (Yamauchi et al. 2016).

A number of herbal plants have been reported to show the variety of bioactivity and investigated the mechanism so far. (Farzaneh and Carvalho 2015; Farzaneh et al. 2018) Medicinal plants in Indonesia have been used as Jamu, a traditional medicine. These medicinal plants are grouped into four categories based on usage, healthcare, beauty care (cosmetic), tonic or beverages, and body protection. (Risman and Roemantyo 2002) *Syzygium polyanthum* (Weight) Walp from the Myrtaceae family is abundantly distributed in Indonesia. *S. polyanthum*, also known as Daun Salam, has been widely used as a culinary additive due to its flavor and its effectiveness in the treatment of diarrhea, diabetes, hypertension, etc. (Noorma et al. 1995; Sumono and SD 2008; Lee et al. 2012; Ismail et al. 2013; Widharna et al. 2015) Our previous study revealed that the ingredients of Salam leaves inhibit melanogenesis in B16 melanoma cells. (Setyawati et al. 2018).

Zingiber officinale var rubrum (red ginger), which is used in Jamu, is a herbaceous perennial belonging to the Zingiberaceae family and is widely distributed in Indonesia, China, India, etc. *Z. officinale* var rubrum is known as Jahe merah in Indonesia and is used for the treatment of diabetes, inflammation, neurologic disease, and asthma. (Chrubasik et al. 2005; Ali et al. 2008; Jayanudin et al. 2015) The high bioactivity of *Z. officinale* var rubrum has been revealed in past studies. The extract of this herb has been reported to show antioxidant activity and anticancer activity (against Hela cells), as well as antibacterial effect against *Bacillus subtilis*. (Philip et al. 2009; Ghasemzadeh et al. 2015). Two vanilloid compounds, 6-gingerol and shogaol, have been isolated from *Z. officinale* var rubrum, and their antioxidant and anticancer activity has been evaluated. (Ghasemzadeh et al. 2015) However, the detail ingredients are still unclear. Then we investigated the structure of ingredients using analytical instruments, and the effects of the ingredients from *Z. officinale* var rubrum on melanogenesis were evaluated using B16 melanoma cells. 6-Shogaol, 6-gingerol, and 8-gingerol have been reported to inhibit melanogenesis. (Huang et al. 2011; Yao et al. 2013; Huang et al. 2013, 2014a) However the structure-activity relationships for the vanilloid compounds were not elucidated yet. Hence, we investigated them using the isolated components and synthesized compounds in this report. This is the first report on the effect of the ingredients from *Z. officinale* var rubrum extract on melanogenesis.

Materials and methods

Plant material

Z. officinale var rubrum was collected from Bogor, West Java, Indonesia, and identified by Research Center for Biology, Indonesia Institute of Sciences, Cibinong, Jakarta. Voucher specimens (No. BMK0098082016) were deposited at Tropical Biopharmaca Research Center, Bogor Agricultural University, Indonesia.

Extraction and fractionation of Z. officinale var rubrum root powder

Z. officinale var rubrum rhizome powder (500 g) was extracted with methanol (2.5 L), and the methanol extract (27.7 g) was extracted with water. The insoluble part was extracted with ethyl acetate to obtain the WIS-ES fraction (14.1 g). The WIS-ES fraction (7.6 g) was separated by silica gel column chromatography (74 mm $\varphi \times 518$ mm L) via stepwise elution from *n*-hexane to ethyl acetate (3:1 to 1:1 v/v) to obtain fractions 1–10 (Fr. 1–10). Fr.7 was separated via silica gel column chromatography (24 mm $\varphi \times 420$ mm L) by elution with *n*-hexane and ethyl acetate (1:1 v/v) to obtain Fr. 7-1–Fr. 7-4. Compounds **1**, **2**, and **11** were isolated from Fr. 7-3 using preparative HPLC [ODS-3 (10 mm $\varphi \times 250$ mm L) (MeOH:0.05% TFA aq. = MeOH:0.05% TFA aq. = 65%:35% (20 min. isocratic) → 75%:25% (10 min) → 75%:25% (8 min. isocratic)] and **3** was isolated from Fr. 7-2 using preparative HPLC [ODS-3 (10 mm $\varphi \times 250$ mm L) (MeOH:0.05% TFA aq. = 70%:30 → 100%:0% (40 min)]. Compound **13** was isolated from Fr. 4 using silica gel column chromatography (23 mm $\varphi \times 430$ mm L) by elution with *n*-hexane and ethyl acetate (5:1 v/v). Compound **12** was isolated from Fr.2 using silica gel column chromatography (23 mm $\varphi \times 450$ mm L) by elution with *n*-hexane and ethyl acetate (9:1 v/v). Fr. 8 was separated via silica gel column chromatography (24 mm $\varphi \times 420$ mm L) by elution with chloroform and methanol (50:1 v/v) to obtain Fr. 8-1–Fr. 8–9. Compound **8** was isolated from Fr. 8-7 using preparative HPLC [ODS-3 (10 mm $\varphi \times 250$ mm L) (MeOH:0.05% TFA aq. = 65%:35 → 100%:0% (40 min)]. Compounds **5**–**7** were isolated from Fr. 3 using preparative HPLC [ODS-3 (10 mm $\varphi \times 250$ mm L) (MeOH:0.05% TFA aq. = 50%:50 → 100%:0% (50 min)]. Compounds **3**, **4**, **9**, and **10** were isolated from Fr. 4 using preparative HPLC [ODS-3 (20 mm $\varphi \times 250$ mm L) (MeOH:0.05% TFA aq. = 70%:30 → 100%:0% (40 min)].

Identification of compounds

Compounds **1**–**13** were identified by ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, HMQC, HMBC, NOESY (Jeol ECA 600 NMR spectrometer, Table S1) and MALDI-TOF-MS (Shimadzu Biotech Axima Resonance 2.9.1.20100121). CD_3OD was used for **3** as the solvents of the NMR experiment, and $CDCl_3$ was used for other isolated compounds.

Compound 1

6-Gingerol: λ_{max} 204, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 317.6394 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 2

8-Gingerol: λ_{max} 204, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 345.2655 *m/z* $[M + Na]^+$ and 361.2337 *m/z* $[M + K]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 3

5-Methoxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone: λ_{max} 204, 224, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 331.2735 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:120).

Compound 4

5-Methoxy-1-(4-hydroxy-3-methoxyphenyl)-3-tetradecanone: λ_{max} 204, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 387.3477 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:130).

Compound 5

6-Shogaol: λ_{max} 204, 228, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 299.6695 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 6

8-Shogaol: λ_{max} 204, 228, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 327.7501 *m/z* $[M + Na]^+$ and 343.7548 *m/z* $[M + K]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 7

10-Shogaol: λ_{max} 228, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 355.8415 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 8

1-(4-Hydroxy-3-methoxyphenyl)-3,5-decanediol: λ_{max} 204, 224, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 319.6328 *m/z* $[M + Na]^+$ and 335.6 *m/z* $[M + K]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 9

3,5-Diacetoxy-1-(4-hydroxy-3-methoxyphenyl)decane: λ_{max} 204, 224, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 403.2774 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 10

4-Gingeracetate: λ_{max} 204, 224, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 375.3246 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:130).

Compound 11

3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-5-decanone: λ_{max} 204, 224, 280 nm; The molecular weight was determined by MALDI-TOF-MS as 317.6358 *m/z* $[M + Na]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

Compound 12

Ethyl cinnamate: λ_{max} 216, 276 nm; The molecular weight was determined by MALDI-TOF-MS as 177.5862 *m/z* $[M + H]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:130).

Compound 13

Ethyl p-methoxycinnamate: λ_{max} 228, 308 nm; The molecular weight was determined by MALDI-TOF-MS as 207.1239 *m/z* $[M + H]^+$ (Shimadzu Biotech Axima Resonance: Mode positive, Low 100+, power:100).

The NMR data of isolated compounds were shown in supplementary material.

Cell culture

Cells were cultured as previously described (Yamauchi et al. 2015a, 2015b) with minor modifications. Murine melanoma B16-F0 cells (DS Pharma Biomedical, Osaka, Japan) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 9% fetal bovine serum (FBS, Biosera, Japan), 100,000 unit/L penicillin, and 100 mg/L streptomycin. The cells were cultured in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air at 37 °C.

Determination of melanin content in B16 melanoma cells

The cellular melanin content was determined as previously described (Yamauchi et al. 2014, 2015b) with minor modifications. In brief, the confluent culture of B16 melanoma cells was raised in phosphate-buffered saline (PBS) and removed using 0.25% trypsin/EDTA. The cells were added to a 24-well plate (0.5×10^5 cells/well) and allowed to adhere at 37 °C for 24 h. Then, the cells were incubated for 72 h. The supernatant (extracellular melanin formation) was collected in an Eppendorf tube. Subsequently, 200 µL of the supernatant was transferred to a 96-well plate, and the absorbance was determined at 510 nm. To determine intracellular, the cells were washed with PBS, followed by lysis in 600 µL of 1N NaOH by heating for 30 min at 100 °C to dissolve melanin. The resulting lysate (250 µL) was added to a 96-well microplate, and the absorbance was measured at 405 nm using a microplate reader.

Cell viability assay

The cell viability was determined as previously described (Yamauchi et al. 2014, 2015b) using the micro culture tetrazolium technique (MTT). Cultures were initiated in 24-well plates at 0.5×10^5 cells per well. After incubation, 50 µL of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-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 of isopropyl alcohol (containing 0.04N HCl) was added to the plate, and 150 µL of the isopropyl alcohol solution was added to a 96-well plate. The absorbance was measured at 590 nm using a microplate reader.

Glucosylation of shogaol

Glucosylation of 6-shogaol was performed as shown in Fig. 1. 6-Shogaol (**5**) (69.9 mg, 0.25 mmol) and acetobromoglucose (329.8 mg, 0.80 mmol) were added to 1600 µL of chloroform, and KOH (44.9 mg, 0.80 mmol) and benzyltributylammonium chloride (249.6 mg, 0.80 mmol) in 2000 µL of water were added. Then, the mixture was stirred for 6.5 h at room temperature. The reaction mixture was diluted with 3 mL of chloroform and washed with water (2 × 4 mL). The chloroform phase was dried using Na_2SO_4, and the reactant was obtained by evaporating the solvent. 4′-*O*-β-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-shogaol (**14**) was purified via silica gel column chromatography (25 mm $\varphi \times 180$ mm L) by elution with *n*-hexane and ethyl acetate (1:1 v/v), and the product was obtained in a yield of 66.4 mg (44.0%). Then, 66.4 mg of **14** (0.11 mmol) was dissolved in 1 mL of MeOH, and K_2CO_3 (41.4 mg, 0.30 mmol) was added. The mixture was stirred for 10 min at room temperature. After neutralization with HCl solution, 4'-*O*-β-glucosyl-6-shogaol (**15**) 3.3 mg (3.0% yield based on 6-shogaol) was purified using preparative HPLC with an ODS-3 column (20 mm φ × 250 mm L) eluted using a linear gradient of MeOH/0.05% TFA aq. soln. = 50/50 (0 min), 100/0 (40 min), 100/0 (50 min). The structures of the synthesized compounds were confirmed by NMR (Fig. S1) and UPLC or MALDI-TOFMS.

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UV λ_{max} 200, 230, 275, 334, 406: MALDI-TOF-MS: *m/z* 630.2689 $[M + Na]^+$; ^{1}H-NMR (Croloform-D, JEOL ECA-600, 600 MHz): δH (ppm) 0.87 (3H, t, $J = 7.2$ Hz, H-10), 1.29 (4H, m, H-8 and H-9), 1.43 (2H, quin, $J = 7.6$ Hz, H-7), 2.01 and 2.06 (12H, s, OAc), 2.18 (2H, m, H-6), 2.82 (2H, m, H-2), 2.87 (2H, m, H-1), 3.72 (1H, m, H-5"), 3.77 (3H, s, 3'-OMe), 4.14 (1H, dd, $J = 2.7$ and 12.4 Hz, H-6"), 4.26 (1H, dd, J = 4.8 and 12.5 Hz, H-6"), 4.89 (1H, d, $J = 7.6$ Hz, H-1"), 5.14 (1H, t, $J = 9.6$ Hz, H-4"), 5.25 (2H, m, H-2" and 3"), 6.08 (1H, d, $J = 15.8$, H-4), 6.67 (1H, dd, $J = 2.0$ and 8.2 Hz, H-6′), 6.73 (1H, d, $J = 2.0$ Hz, H-2′), 6.81 (1H, m, H-5), 7.00 (1H, d, $J = 8.3$ Hz, H-5'); ^{13}C-NMR (Croloform-D, 150 MHz) δc (ppm) 14.0 (C-10), 20.6, 20.7 × 2 and 20.8 (OAc), 22.5 (C-8), 31.4 (C-9), 27.8 (C-7), 29.8 (C-1), 32.5 (C-6), 41.7 (C-2), 56.1 (3'-OMe), 62.0 (C-6"), 68.5 (C-4"), 71.3 and 72.7 (C-2", C-3"), 77.1 (C-5"), 101.1 (C-1"), 113.2 (C-2′), 120.4 × 2 (C-5′ and C-6′), 130.3 (C-4), 138.3 (C-1′), 144.4 (C-4′), 148.0 (C-5), 150.6 (C-3′), 169.4, 170.3 × 2, 170.7(OAc)199.5 (C-3)

Compound 15

UV λ_{max} 222, 204, 275, 486, 406: MALDI-TOF-MS: *m/z* 461.1219 $[M + Na]^+$; 1H-NMR (Methanol-4D, JEOL ECA-600, 600 MHz): δH (ppm) 0.89 (3H, t, $J = 6.9$ Hz, H-10), 1.27–1.34 (4H, m, H-8 and H-9), 1.45 (2H, quin, $J = 7.4$ Hz, H-7), 2.20 (2H, q, $J = 7.3$ Hz, H-6), 2.58 (2H, m, H-1), 2.87 (2H, m, H-2), 3.37 (2H, m, H-4" and H-5"), 3.42 (1H, t, $J = 8.9$ Hz, H-3"), 3.45 (1H, t, $J = 8.3$ Hz,H-2"),

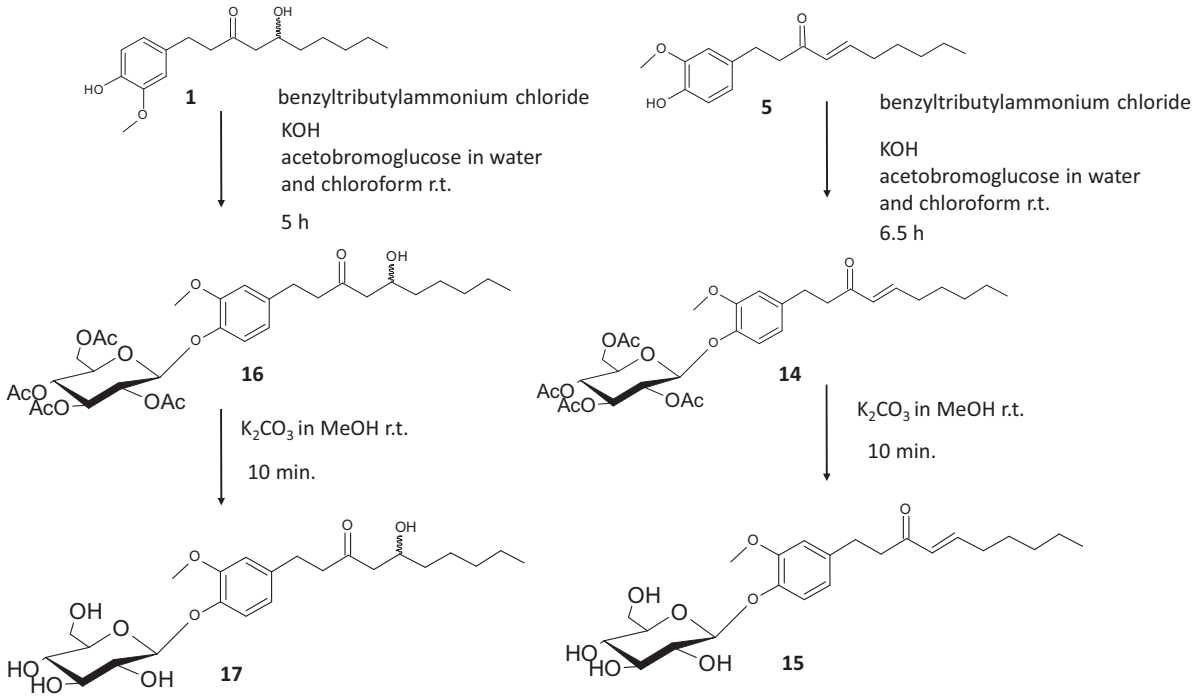

Fig. 1 Synthesis scheme of glucosylated 6-gingerol and shogaol

3.66 (1H, dd, $J = 5.2$ and 12.0 Hz, Ha-6"), 3.82 (3H, s, 3'-OMe), 3.84 (1H, dd, $J = 1.4$ and 12.4 Hz, Hb-6"), 4.80 (1H, d, $J = 7.6$ Hz, H-1"), 6.69 (1H, d, $J = 16.5$ Hz, H-4), 6.71 (1H, dd, $J = 2.1$ and 8.3 Hz, H-6'), 6.84 (1H, d, $J = 2.0$ Hz, H-2′), 6.89 (1H, sext, $J = 6.9$ Hz, H-5), 7.04 (1H, d, $J = 8.2$ Hz, H-5′); ^{13}C-NMR (Methanol-4D, JEOL ECA-600, 150 MHz) δc (ppm) 13.0 (C-10), 22.1 and 31.2 (C-8 and C-9), 27.6 (C-7), 29.6 (C-1), 32.2 (C-6), 41.1 (C-2), 55.4 (3'-OMe), 61.2 (C-6"), 70.0 (C-5"), 73.6 (C-3"), 76.5 (C-2"), 76.8 (C-4"), 101.7 (C-1"), 112.7 (C-2′), 116.9 (C-5′), 120.5 (C-6′), 129.9 (C-4), 136.3 (C-1′), 145.0 (C-4′), 148.7 (C-3′), 149.4 (C-5), 201.1 (C-3)

Glucosylation of gingeol

Glucosylation of 6-gingerol was performed as shown in Fig. 1. 6-Gingeol (**1**) (73.7 mg, 0.25 mmol) and acetobromoglucose (164.5 mg, 0.40 mmol) were added to 800 µL of chloroform, and KOH (22.4 mg, 0.40 mmol) and benzyltributylammonium chloride (249.6 mg, 0.80 mmol) in 600 µL of water were added. Then the mixture was stirred for 5 h at room temperature. The reaction mixture was diluted with 3 mL of chloroform and washed with water (2 × 4 mL). The chloroform phase was dried using Na_2SO_4, and the reactant was obtained by evaporating the solvent. 4′-*O*-β-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-gingeol (**16**) was purified via silica gel column chromatography (18 mm φ × 280 mm L) by the elution of *n*-hexane and ethyl acetate (1:1 v/v), and the product was obtained in a yield of 62.1%. Then, 77.6 mg of **16** (0.12 mmol) was dissolved in 1 mL of MeOH and K_2CO_3 (33.2 mg, 0.2 mmol) was added. The mixture was stirred for 20 min at room temperature. After neutralization with HCl solution, 4'-*O*-β-glucosyl-6-gingerol (**17**) 32.0 mg (28.0% yield based on 6-gingeol) was purified via silica gel column chromatography (16 mm φ × 120 mm L) by the elution of *n*-hexane and ethyl acetate (3:1 v/v). The structures of the synthesized compounds were confirmed by NMR (Fig. S2) and UPLC or MALDI-TOFMS.

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UV λ_{max} 205, 218, 274, 485, 579: MALDI-TOF-MS: *m/z* 647.4890 $[M + Na]^+$; ^{1}H-NMR (Chloroform-D, JEOL ECA-600, 600 MHz): δH (ppm) 0.86 (3H, t, $J = 6.9$ Hz, H-10), 1.23-1.32 (6H, m, H-7, 8, and 9), 1.34–1.48 (2H, m, H-6), 2.01, 2.02, 2.06 (12H, s, OAc), 2.48 (1H, dd, $J = 9.3$ and 17.6 Hz, Ha-4), 2.56 (1H, dd, J = 2.7 and 17.2 Hz, Hb-4), 2.73 (2H, t, $J = 7.2$ Hz, H-2), 2.83 (2H, t, $J = 7.5$ Hz, H-1), 3.73 (1H, m, H-5"), 3.77 (3H, s, 3'-OMe), 4.01 (1H, m, H-5), 4.14 (1H, dd, $J = 2.8$ and 12.4 Hz, Ha-6"), 4.26 (1H, dd, $J = 5.5$ and 12.4 Hz, Hb-6"), 5.07 (1H, d, $J = 8.3$ Hz, H-1"), 5.14 (1H, t, $J = 9.6$ Hz, H-4"), 5.25 (2H, m, H-2" and H-3"), 6.64 (1H, dd, $J = 2.1$ and 8.3 Hz, H-6′), 6.70 (1H, d, $J = 2.3$ Hz, H-2′), 7.00 (1H, d, $J = 8.2$ Hz, H-5′); ^{13}C-NMR (Chloroform-D, JEOL ECA-600, 150 MHz) δc (ppm) 14.1 (C-10), 20.7 × 3 and 20.8 (OAc), 22.7, 25.2 and 31.8 (C-7, C-8, and C-9), 29.2 (C-1), 36.5 (C-6), 45.1 (C-2),

49.4 (C-4), 56.1 (3'-OMe), 62.0 (C-6"), 68.5 (C-4"), 71.3 and 72.7 (C-2" and C-3"), 72.0 (C-5"), 101.1 (C-1"), 113.0 (C-2′), 120.3 (C-6′), 120.5 (C-5′), 137.7 (C-1′), 144.5 (C-4′), 150.7 (C-3′), 169.5 × 2, 170.4, and 170.7 (C-OAc), 211.1 (C-3)

Compound 17

UV λ_{max} 201, 220, 276, 486, 400: MALDI-TOF-MS: *m/z* 479.3252 $[M+Na]^+$; ^{1}H-NMR (Methanol-D_4, JEOL ECA-600, 600 MHz): δH (ppm) 0.89 (3H, t, $J = 6.9$ Hz, H-10), 1.24–1.42 (8H, m, H-6, -7, -8 and -9), 2.47 (1H, dd, $J = 4.8$ and 15.8 Hz, Ha-4), 2.53 (1H, dd, $J = 8.3$ and 15.8 Hz, Hb-4), 2.78 (4H, m, H-1 and -2), 3.31-3.38 (2H, m, H-4" and H-5"), 3.42 (1H, t, $J = 8.4$ Hz, H-3"), 3.45 (1H, t, $J = 8.3$ Hz, H-2"), 3.64-3.85 (2H, m, H-6"), 3.82 (3H, s, 3'-OMe), 3.98 (1H, m, H-5), 4.80 (1H, d, $J = 7.6$ Hz, H-1"), 6.71 (1H, dd, $J = 2.1$ and 8.3 Hz, H-6′), 6.84 (1H, d, $J = 2.0$ Hz, H-2′), 7.04 (1H, d, $J = 8.2$ Hz, H-5′); ^{13}C-NMR (Methanol-D_4, JEOL ECA-600, 150 MHz): δc (ppm) 13.0 (C-10), 22.3 and 31.6 (C-8 and C-9), 25.0 and 37.1 (C-6 and C-7), 28.8 (C-1), 44.8 (C-2), 49.9 (C-4), 55.3 (3'-OMe), 61.2 (C-6"), 67.6 (C-5), 70.0 and 76.8 (C-4" and C5"), 73.5 (C-3"), 76.5 (C-2"), 101.7 (C-1"), 112.7 (C-2′), 116.9 (C-5′), 120.5 (C-6′), 136.4 (C-1′), 144.9 (C-4′), 149.4 (C-3′), 210.3 (C-3)

Statistical analysis

Cell viability and melanogenesis activity have been expressed as mean ± SD values. Differences were examined for statistical significance by using the Student's *t*-test.

Results and discussion

Thirteen compounds, including eleven vanilloid compounds, the gingerol and shogaol derivatives, were isolated and identified from *Z. officinale* var rubrum rhizome extract (Fig. 2). The high bioactivity of the vanilloid compounds has been clarified earlier. Vanilloid compounds have also been isolated from the seeds of *Aframomum melegueta*, which is widely distributed in West Africa. It has been used as a spice and a folk remedy, and its extract shows anti-obesity effect in mice. (Hattori et al. 2017, 2018) 6 and 8-Gingerol inhibits melanogenesis by downregulation of the intracellular reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) and protein kinase A

Compounds	R^1	R^2	R^3
1	CH_3	OH	
2	$(CH_2)_2CH_3$	OH	
3	CH_3	OCH_3	
4	$(CH_2)_4CH_3$	OCH_3	
5			CH_3
6			$(CH_2)_2CH_3$
7			$(CH_2)_4CH_3$

Compounds	R^4	R^5	R^6	R^7
8	$(CH_2)_2CH_3$	OH	OH	
9	$(CH_2)_2CH_3$	OAc	OAc	
10	CH_3	OAc	OAc	
11	$(CH_2)_2CH_3$	=O	OH	
12				H
13				OCH_3

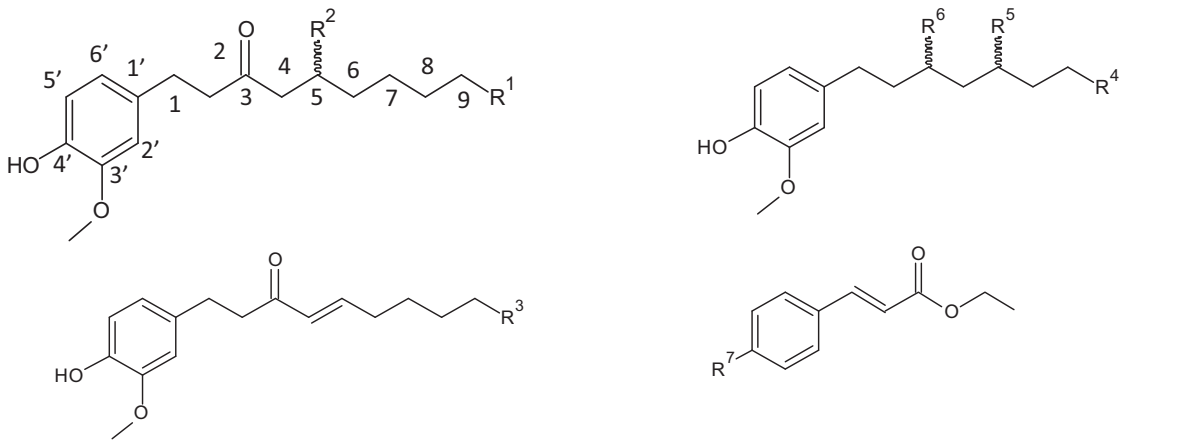

Fig. 2 Structures of isolated compounds from *Zingiber officinale* var rubrum

Table 1 Intracellular and extracellular melanogenesis levels and cell viabilities of **1**–**13**, **15**, and **17**

Compounds (μM)	Cell viability (%)	Intracellular melanogeneis level (%)	Extracellular melanogenesis level (%)	Compounds (μM)	Cell viability (%)	Intracellular melanogeneis level (%)	Extracellular melanogenesis level (%)
1				**9**			
100.0	36 ± 3.7**	99 ± 0.9	31 ± 0.1**	100.0	17 ± 2.7**	53 ± 2.0*	35 ± 1.1**
50.0	73 ± 5.1*	109 ± 1.8	52 ± 0.2**	50.0	39 ± 2.9**	83 ± 1.8	37 ± 0.2**
25.0	88 ± 0.2*	111 ± 2.4	83 ± 5.5	25.0	65 ± 3.8**	111 ± 11.3	46 ± 5.9**
12.5	92 ± 3.8	103 ± 6.7	82 ± 2.8*	12.5	88 ± 1.9	110 ± 2.6	59 ± 4.2*
2				**10**			
100.0	25 ± 3.9**	93 ± 1.8	27 ± 1.1**	100.0	68 ± 2.0**	104 ± 2.9	41 ± 1.9**
50.0	80 ± 3.5*	92 ± 3.5	37 ± 1.8**	50.0	78 ± 1.0*	111 ± 0.4	58 ± 4.2*
25.0	88 ± 6.1	107 ± 0.2	64 ± 4.7*	25.0	86 ± 0.6*	119 ± 0.2	80 ± 8.2
12.5	84 ± 3.2*	104 ± 2.3	78 ± 6.0	12.5	90 ± 4.1	114 ± 1.1	96 ± 1.4
3				**11**			
100.0	42 ± 4.5**	58 ± 3.0**	43 ± 0.2**	100.0	13 ± 0.1**	50 ± 2.1**	33 ± 0.2**
50.0	56 ± 6.1*	81 ± 1.4**	42 ± 1.2**	50.0	68 ± 2.2**	106 ± 0.5*	40 ± 0.1**
25.0	67 ± 0.5**	96 ± 1.7	47 ± 0.1**	25.0	90 ± 1.7	117 ± 1.0**	61 ± 2.4**
12.5	91 ± 5.9	100 ± 0.9	63 ± 3.3*	12.5	96 ± 1.8	119 ± 2.4**	82 ± 6.0
4				**12**			
50.0	59 ± 4.1**	60 ± 0.9**	35 ± 0.6**	200.0	70 ± 2.4	70 ± 3.5**	43 ± 0.3**
25.0	61 ± 5.7**	84 ± 0.5*	39 ± 0.2**	100.0	110 ± 2.1	87 ± 2.7*	89 ± 4.3
12.5	64 ± 2.9**	103 ± 0.8	41 ± 1.2**	50.0	80 ± 1.4	96 ± 1.0*	106 ± 4.5
6.25	68 ± 2.8**	115 ± 4.2	51 ± 1.3**	25.0	84 ± 5.9	99 ± 1.5	103 ± 0.5
5				**13**			
50.0	45 ± 4.9*	54 ± 3.2**	78 ± 8.9	200.0	57 ± 0.6*	84 ± 1.0**	53 ± 3.5**
25.0	80 ± 6.9	72 ± 0.2*	110 ± 2.0*	100.0	66 ± 1.5*	89 ± 1.2**	69 ± 5.0*
12.5	88 ± 0.5	78 ± 2.1*	106 ± 3.1	50.0	77 ± 4.0	91 ± 1.7*	84 ± 3.4*
6.25	97 ± 6.6	87 ± 5.5	112 ± 1.3**	25.0	87 ± 6.1	96 ± 1.9	98 ± 2.0
6				**15**			
50.0	51 ± 3.3**	47 ± 2.1**	57 ± 9.3*	50.0	71 ± 2.3*	80 ± 5.9	55 ± 12.7*
25.0	73 ± 4.1*	55 ± 1.2**	92 ± 2.3*	25.0	75 ± 0.1*	86 ± 0.5	68 ± 5.0*
12.5	93 ± 0.9	65 ± 0.8*	110 ± 1.2**	12.5	78 ± 3.3*	91 ± 2.0	77 ± 8.2
6.25	109 ± 0.8	77 ± 1.6*	110 ± 0.5**	6.25	81 ± 5.0	93 ± 5.3	91 ± 9.9
7				**17**			
50.0	48 ± 2.9**	40 ± 1.1**	23 ± 1.7**	50.0	84 ± 0.6*	101 ± 1.6	101 ± 0.6
25.0	58 ± 0.6**	43 ± 0.2**	35 ± 2.4**	25.0	100 ± 3.2	100 ± 3.0	93 ± 0.9
12.5	76 ± 0.7**	55 ± 2.3**	90 ± 2.9*	12.5	101 ± 3.1	104 ± 1.0	94 ± 1.5
6.25	105 ± 2.5	72 ± 1.6**	114 ± 0.5**	6.25	96 ± 5.7	105 ± 0.1	106 ± 3.0
8							
400.0	35 ± 2.1**	54 ± 1.2**	35 ± 0.2**				
200.0	107 ± 17.8	84 ± 6.2	96 ± 1.2	Arbutin			
100.0	94 ± 1.1	89 ± 1.0**	105 ± 0.1	730.0	121 ± 2.7	77 ± 6.1*	42 ± 1.5**
50.0	101 ± 6.5	88 ± 1.0**	99 ± 6.0				

All data were expressed as the mean ± S.D. $n = 2$

*$p \le 0.05$ and **$p \le 0.01$ compared with respective control values

(PKA) signaling pathways. (Huang et al. 2011; Huang et al. 2013) Furthermore, 6-shogaol inhibits melanogenesis through the stimulation of extracellular responsive kinase (ERK) and phosphatidylinositol-3-kinase (PI3K/Akt) that degrade MITF. (Yao et al. 2013; Huang et al. 2014b) However, the structure-activity relationship for the vanilloid

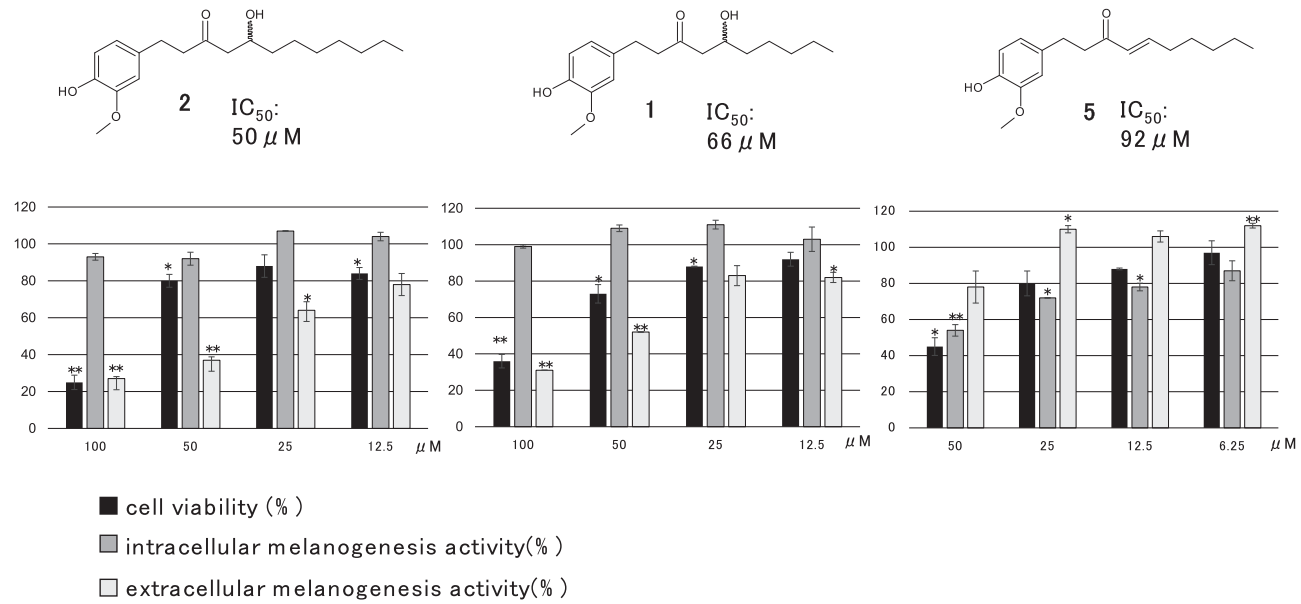

Fig. 3 Cell viability, intracellular melanogenesis activity, and extracellular melanogenesis activity of **1**, **2**, and **5**. IC_{50}: The concentration of the compounds that shows 50% extracellular melanognesis inhibition. All data were expressed as the mean ± S.D. $n = 2$. $*p \le 0.05$ and $**p \le 0.01$ compared with respective control values

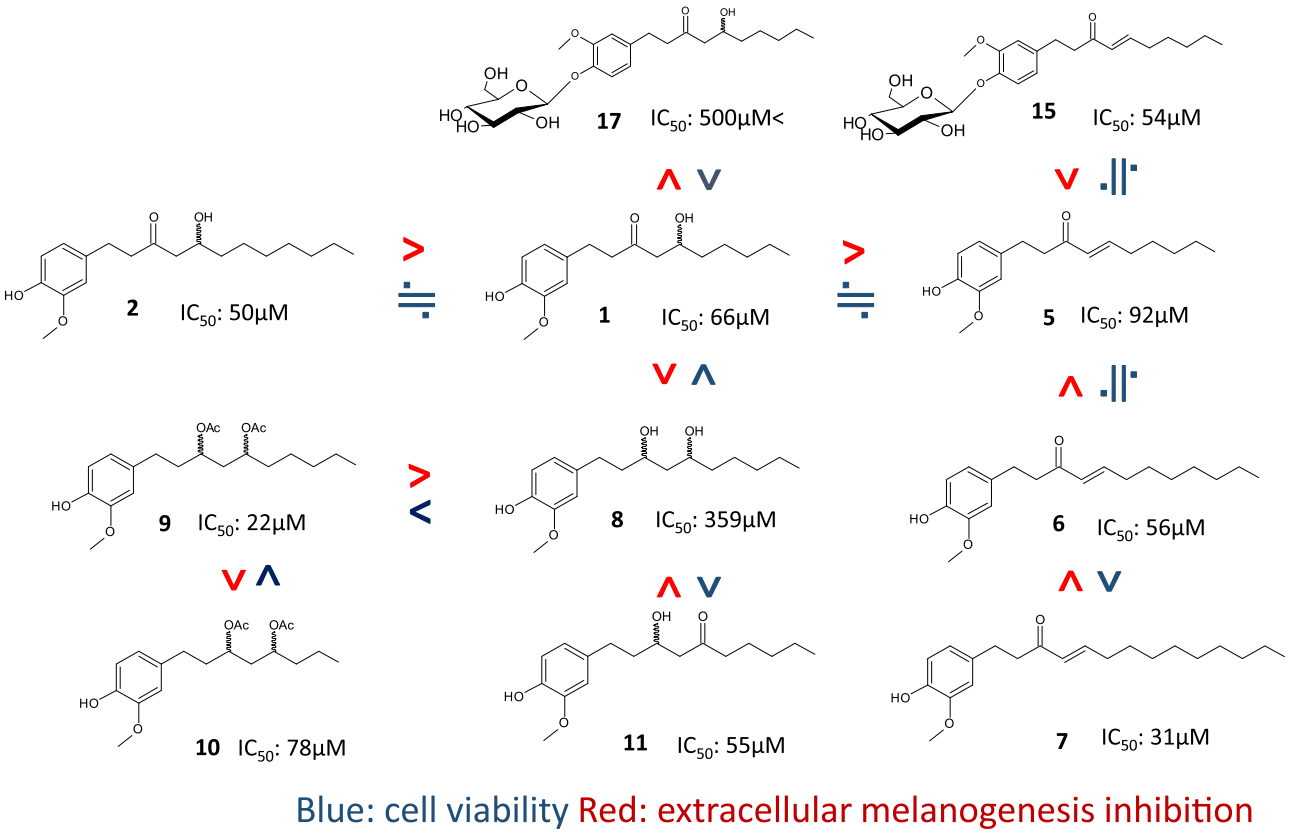

Fig. 4 Structure activity relationships of vanilloid compounds on extracellular melanogenesis inhibitory activity. IC_{50}: Concentrations of the compounds that shows 50% extracellular melanogenesis inhibition

compounds regarding the melanogenesis inhibitory activity is still unclear. Moreover, the inhibitory effect is limited to intracellular melanogenesis. Considering that the extracellular cellular melanin content is approximately 23 times higher than the intracellular melanin content, 25 the effect on extracellular melanogenesis should be evaluated. Hence, we investigated the structure-activity relationships using gingerol and shogaol derivatives for the extracellular melanogenesis inhibitory activity in B16 melanoma cells. Shogaols (**5**–**7**) inhibited intracellular and extracellular melanogenesis. However, 6-gingerol (**1**) and 8-gingerol (**2**) did not bring about a decrease in the intracellular melanin content, although they inhibited extracellular melanogenesis. The results indicate that the gingerols inhibited not only melanogenesis but also the transportation of melanosome outside the cells, and that the mechanisms underlying the melanogenesis inhibitory activity of shogaol and gingerol were different.

The extract of *Z. officinale* var rubrum including the vanilloid compounds inhibited melanogenesis. We also evaluated the effect of the components in the extract on the melanogenesis. Gingerol derivatives (**1**–**4**) inhibited extracellular melanogenesis in a dose-dependent manner. Among them, **2** exhibited 64% extracellular melanogenesis activity with high cell viability at 25 μM, and **3** inhibited extracellular melanogenesis to 63% with low cell toxicity at 12.5 μM (Table 1 and Fig. 3). The structure activity relationships of vanilloid compounds and IC_{50} value which are concentrations of the compounds that shows 50% extracellular melanogenesis inhibition were shown in Fig. 4. Compound **2** (IC_{50}:50 μM) inhibited extracellular melanogenesis in a more potent manner than did **1** (IC_{50}:66 μM) at the similar cell viability. The effect of **8** (IC_{50}:359 μM) was lower than that of **2**. These results suggested that the length of the carbon chain and the carbonyl group in the gingerol were involved in the inhibitory effect. Acetylation of the hydroxyl groups in **8** decreased the cell viability. Elongation of the carbon chain in the **9** (IC_{50}:22 μM) and **10** (IC_{50}:78 μM) also resulted in increased extracellular melanogenesis inhibitory activity. Among the shogaol derivatives (**5**–**7**), **7** (IC_{50}:31 μM) showed higher extracellular melanogenesis inhibitory activity than did **5** (IC_{50}:92 μM) and **6** (IC_{50}:56 μM). Compound **6** inhibited extracellular melanogenesis in a more potent manner than did **5**, indicating that the elongation of the carbon chain in shogaol increased the inhibitory activity. The effect of 6-shogaol (**5**) was weaker than that of 6-gingerol (**1**) at the similar cell viability, which suggested that the carbonyl and hydroxyl groups in 6-gingerol play an important role in accelerating the effect.

In order to evaluate the importance of the vanillyl group in the compounds isolated from *Z. officinale* var rubrum rhizome, we glycosylated the phenolic hydroxyl group in 6-gingerol and shogaol using benzyltributylammonium chloride as the catalyst in a mixed solvent of chloroform and water (Fig. 1). The synthesized compounds were confirmed by NMR and MALDI-TOFMS. The combined position of glucose was confirmed by the HMBC correlation between the anomeric proton in glucose and the 4'-carbon in 6-gingerol and shogaol. Interestingly, glycosylation of 6-shogaol increased the extracellular melanogenesis inhibitory activity, even though glycosylation of 6-gingerol nullified the activity (Fig. 4). This result suggested that the mechanism underlying the inhibitory effect for gingerol and shogaol is different: the vanillyl group is necessary for the activity in the case of gingerol but not in the case of shogaol. Or it may be relating the permeability of the compounds to the cell membrane. Additional data would be necessary to clarify the detailed mechanism underlying the melanogenesis inhibitory activity of the vanilloid compounds. However, the fundamental information regarding the structure-activity relationship of the vanilloid compounds could aid the development of whitening agent as cosmetic products.

Conclusion

Eleven vanilloid compounds and two cinnamic acid derivatives were isolated and identified from the methanol extract of *Z. officinale* var rubrum rhizome. 6 and 8-Gingerol and 6-shogaol are known to be melanogenesis inhibitors. Structure-activity relationships were investigated using the components in the *Z. officinale* var rubrum extract. Elongation of the carbon chain of gingerol and shogaol enhanced the extracellular melanogenesis inhibitory activity. Moreover, the hydroxyl and carbonyl groups on the carbon chain of gingerol increased the activity. Glycosylation of the phenolic hydroxyl group of shogaol improved the melanogenesis inhibitory activity, but glycosylation of 6-gingerol nullified the extracellular melanogenesis inhibitory activity. The results of this provide fundamental information for the development of whitening agents based on vanilloid compounds.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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