

Melanin Biosynthesis Inhibitory and Antioxidant Activities of Quercetin-3'-O-β-D-glucoside Isolated from *Allium cepa*

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In the course of searching for new whitening agents, we have found that the methanol extract of dried skin of *Allium cepa* shows potent melanin biosynthesis inhibitory activity in B16 melanoma cells. Bioassay-guided fractionation led to the isolation of quercetin-3'-O-β-D-glucoside (**1**) from the methanol extract of dried skin of *A. cepa*, which inhibited melanin formation in B16 melanoma cells with an IC₅₀ value of 38.8 μM and mushroom tyrosinase with an IC₅₀ value of 6.5 μM using L-tyrosine and 48.5 μM using L-dihydroxyphenylalanine as substrates, respectively. In addition, the antioxidant activity of **1** was evaluated in the oxygen radical absorbance capacity assay; it showed 3.04 μmol Trolox equivalents/mmol. **1** was shown to be a promising ingredient that could be useful for treating hyperpigmentation and for protecting against oxidative stress.

Key words: Quercetin-3'-O-β-D-glucoside, Antimelanogenesis, Antioxidant

Introduction

Melanin pigments are formed in specialized pigment-producing cells known as melanocytes, which originate in the neural crest during embryogenesis and are distributed throughout the embryo during its development (Sánchez-Ferrer *et al.*, 1995). Melanin biosynthesis occurs in a cascade of enzymatic and spontaneous reactions that convert tyrosine to melanin pigments. The initial and rate-limiting step in melanin synthesis is the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) (Yoon *et al.*, 2007). DOPA oxidation produces a highly reactive intermediate that is further oxidized to form melanin by a free radical-coupling pathway. If free radicals are inappropriately processed in melanin synthesis, then hydrogen peroxide (H₂O₂) is generated, leading to the production of hydroxyl radicals (HO·) and other reactive oxygen species (ROS) (Perluigi *et al.*, 2003).

Onion (*Allium* species) is a versatile vegetable, and many epidemiological studies have suggested

that regular consumption of onions is associated with a reduced risk of neurodegenerative disorders, cancer, cataract, ulcer, osteoporosis, vascular and heart diseases by inhibition of lipid peroxidation and lowering of low-density lipoprotein cholesterol levels (Kaneko and Baba, 1999; Kawaii *et al.*, 1999; Sanderson *et al.*, 1999; Shutenko *et al.*, 1999). Onion is one of the major sources of various biologically active phytochemicals, *e.g.* phenolic acids, flavonoids, cepaenes, thiosulfonates, and anthocyanins (Singh *et al.*, 2009). The major flavonoids found in dry onion-skin, which have been usually considered waste, contain large amounts of quercetin, quercetin glycoside, and their oxidative products which are effective antioxidants against the lethal effect of oxidative stress (Gulsen *et al.*, 2007; Prakash *et al.*, 2007). In Indonesia the use of onion plays an important role in traditional medicine; it is used as a diuretic and a poultice to cure wounds, reduce the appearance of scars in the skin, suppress the blood sugar level, act as a febrifuge, and prevent platelet aggregation (de Padua *et al.*, 1999).

Based on our preliminary screening data (not shown), the methanol extract of dried skin of red onion (*Allium cepa*) from Indonesia showed potent melanin biosynthesis inhibitory activity on B16 melanoma cells. We therefore focused on the active compound from dried red onion-skin.

Material and Methods

Chemicals

NaOH and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), ethylenediaminetetraacetic acid (EDTA) from Dojindo Co. (Kumamoto, Japan), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, MO, USA), and Eagle's minimum essential medium (EMEM) from Nissui Chemical Co. (Osaka, Japan). Fluorescein sodium salt (FL), 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The other chemicals were of the highest commercially available grade.

Plant materials

Red onion (*A. cepa*) was purchased from a traditional market in Jakarta, Indonesia, in September 2008. A voucher specimen (ETA-CW-6) was deposited at Wood Chemistry Laboratory, Department of Forest Product Technology, Faculty of Forestry, Mulawarman University, Samarinda, Indonesia.

Preparation of plant extracts

The plant materials were dried at room temperature and powdered. The dried materials (17.38 g) were extracted with methanol at room temperature with shaking at 150 rpm during 48 h. The extract solution was filtered and concentrated *in vacuo*, to obtain the crude methanol extract (1.75 g).

Isolation of quercetin-3'-*O*- β -D-glucoside (**1**)

The crude extract of *A. cepa* (1.4 g), which showed a potent inhibitory effect on melanin production in B16 melanoma cells, was applied onto a silica gel column [71 g of Wakogel C-200 (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 3.5 x 50 cm] eluted with *n*-hexane/EtOAc [10:0 (100 ml), 9:1 (50 ml), 7:3 (50 ml), 5:5 (200 ml), 3:7

(200 ml), 1:9 (100 ml)] and EtOAc/MeOH [9:1 (100 ml), 8:2 (100 ml), 7:3 (250 ml), 6:4 (50 ml), 5:5 (100 ml), 4:6 (50 ml), 3:7 (50 ml), 2:8 (50 ml), 1:9 (100 ml), 0:10 (100)] to give 33 fractions (Fr. 1 to Fr. 33). Fraction 23 was subjected to preparative HPLC. The elution of the column (Inertsil Prep-ODS, 20 mm i.d. x 250 mm) with MeOH/H₂O [0.1% trifluoroacetic acid (TFA), 40:60, 5 ml/min] yielded compound **1** (6.9 mg). Using analytical HPLC and NMR spectroscopy, it was analysed and identified as quercetin-3'-*O*- β -D-glucoside (**1**) by comparison with previous NMR data (Wu *et al.*, 2008). The NMR spectra (¹H, ¹³C, HMQC, and HMBC) of the compound were recorded at 400 MHz on a JNM-AL400 FT NMR spectrometer (JEOL Ltd., Tokyo, Japan). The compound was dissolved in DMSO-*d*₆ and chemical shifts were referred to the deuterated solvent.

Tyrosinase enzyme assay

The tyrosinase activity was determined as previously described (Arung *et al.*, 2007). Briefly, all samples were dissolved in DMSO and used for the actual experiment at 30 times dilution. First, 333 μ l of 330 μ M L-tyrosine or 200 μ M L-dihydroxy-phenylalanine (L-DOPA) solution were mixed with 600 μ l of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C. Then, 33 μ l of the sample solution and 33 μ l of the aqueous solution of mushroom tyrosinase (1380 units/ml) were added to the mixture, and the increase in optical density at 475 nm, on the basis of the formation of DOPochrome, was measured. The reaction solution was incubated at 25 °C for 3 min for L-DOPA and 10 min for L-tyrosine, and the absorbance at 475 nm was measured before and after incubation. The reaction was started by addition of the enzyme. Since tyrosinase catalyzes the reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Controls without inhibitor were routinely carried out. Each experiment was carried out in duplicate or triplicate. Kojic acid was used as a positive control.

Oxygen radical absorbance capacity (ORAC) assay

Samples were directly dissolved in acetone/water/acetic acid (70:29.5:0.5, v/v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Trolox, FL, and AAPH solutions were prepared with 75 mM phosphate buffer (pH 7.4).

The ORAC assay was performed as described by Ou *et al.* (2001) with some modification as follows: 300 μ l of the standard (Trolox) or sample solution were mixed with 1.8 ml of 48 nM FL solution, and then incubated independently at 37 °C for 15 min. AAPH solution (900 μ l, 12.9 mM final concentration) was added to the mixture and vortexed for 10 s. The mixture was then immediately placed in a fluorescence spectrophotometer (Model FP-6500, JASCO Co., Ltd., Tokyo, Japan), and the reaction was measured every 5 s for 60 min at 37 °C (excitation wavelength, 485 nm; emission wavelength, 520 nm). A blank (FL + AAPH) using phosphate buffer, standard solutions (6.25–50 μ M Trolox), and sample solutions was measured at the same conditions. Three independent assays were performed for each sample. The area under the fluorescence decay curve (*AUC*) was calculated as

$$AUC = 1 + \sum \frac{f_i}{f_0},$$

where f_0 is the initial fluorescence reading at 0 min, and f_i is the fluorescence reading at time i .

The ORAC values were calculated according to Prior *et al.* (2003) with slight modification. The *netAUC* corresponding to the sample was calculated by subtracting the *AUC* corresponding to the blank. The linear relationship between net area and antioxidant concentration was calculated using Trolox at different concentrations (6.25–50 μ M). The regression of *netAUC* of Trolox on different concentrations was calculated as follows: $Y = 0.4499X - 2.6812$, where X is the *netAUC* of Trolox and Y the Trolox concentration (μ M).

The relative ORAC value was calculated by the following equation: relative ORAC value (μ M TE/ μ mol) = $(0.4499netAUC_{\text{sample}} - 2.6812) / [\text{sample concentration } (\mu\text{M})]$.

Cell culture

A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

Inhibitory effect of melanin biosynthesis using cultured B16 melanoma cells

This assay was conducted as described by Arung *et al.* (2007). Briefly, confluent cultures of

B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the plastic using 0.25% trypsin/EDTA. The cells were placed in two 24-well plastic culture plates (one plate was for determination of melanin content and the other for determination of cell viability) at a density of $1 \cdot 10^5$ cells/well and incubated for 24 h in medium prior to being treated with the samples. After 24 h, the medium was replaced with 998 μ l of fresh medium, and 2 μ l of DMSO were added with or without (control) the test sample at various concentrations ($n = 3$); arbutin was used as a positive control. The cells were incubated for an additional 48 h, and the medium was then replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed (see below).

Determination of melanin content in B16 melanoma cells

The melanin content of the cells after treatment was determined as follows. After removing the medium and washing the cells with PBS, the resulting cell pellet was dissolved in 1.0 ml of 1 M NaOH. The crude cell extracts were assayed using a microplate reader (Bio-Tek, Winooski, VT, USA) at 405 nm to determine the melanin content. The results of the test samples were analysed as the percentage versus control.

Cell viability

Cell viability was determined by use of the microculture tetrazolium technique assay. This assay uses MTT reagent to provide a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. Culture was initiated in 24-well plates at $1 \cdot 10^5$ cells/well. After incubation, 50 μ l MTT reagent [in PBS (5 mg/ml)] were added to each well. The plates were incubated in a humidified atmosphere of 5% of CO₂ at 37 °C for 4 h. After the medium was removed, 1.0 ml isopropyl alcohol (containing 0.04 M HCl) was added into the plate, and the absorbance was measured at 570 nm relative to 630 nm.

Results and Discussion

Isolation of quercetin-3'-*O*- β -D-glucoside (1)

The extract of dried onion-skin dose-dependently inhibited melanin formation in B16 mela-

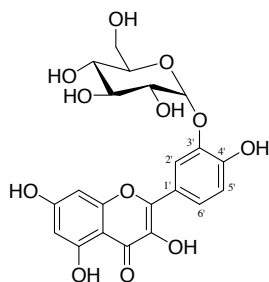


Fig. 1. Chemical structure of quercetin-3'-*O*- β -D-glucoside.

noma cells (data not shown), which led us to isolate the active compound in this extract. As the result of fractionation, fraction 23 seemed to be more potent to inhibit melanin formation in B16 melanoma cells. By preparative HPLC, an active compound was isolated. The NMR assignment was performed to elucidate the structure of this compound by comparison with previous data (Wu *et al.*, 2008), and revealed that this compound was quercetin-3'-*O*- β -D-glucoside (**1**) (Fig. 1). This compound was reported to be isolated from some plants, for example flowers of *Abelmoschus manihot* (Lai *et al.*, 2009), bulbs and flowers of *Hymenocallis littoralis* (Abou-Donia *et al.*, 2008), and flowers of *Gossypium hirsutum* (Wu *et al.*, 2008).

Antimelanin biosynthesis properties

The isolated compound **1** was tested for antimelanogenesis using B16 melanoma cells. The melanin inhibitory effect of **1** is shown in Table I. The activity of **1**, in terms of IC_{50} value, was 38.8 μM with 92% of cell viability at the concentration of IC_{50} , indicating that **1** is more potent than arbutin, the positive control (IC_{50} 198.3 μM). Arbutin is

Table I. Effect of quercetin-3'-*O*- β -D-glucoside on melanin formation (IC_{50}) and cell viability in B16 melanoma cells.

Compound	IC_{50} [μM]	Cell viability (% vs. control) ^a
Quercetin-3'- <i>O</i> - β -D-glucoside (1)	38.8	92.0
Arbutin (positive control)	198.3	95.0

^a Cell viability (%) at the concentration of IC_{50} for melanin formation on B16 melanoma cells. Values show the mean \pm SD ($n = 3$).

used as an active ingredient in cosmetic products, such as Shiseido Whitess (Ando *et al.*, 2010). Only a few of the biological activities of compound **1** have been reported, such as its protective effects on acute myocardial ischemia in mice (Liu and Chen, 2008) and antioxidant effects (Alluis and Dangles, 2001). Here, we found a new facet of the biological activity of **1**, melanin biosynthesis inhibitory activity. Some glycoside flavonoids, such as (+)-taxifolin-3-*O*- β -D-xylopyranoside, (+)-taxifolin-3-*O*- α -L-arabinofuranoside, quercetin-3-*O*- α -L-arabinofuranoside, and tiliroside did not significantly decrease the melanin content of B16 melanoma cells (Fujii and Saito, 2009).

Next, we evaluated the tyrosinase inhibitory activity of compound **1**. The data in Table II shows that compound **1** inhibited mushroom tyrosinase activity with an IC_{50} value of 6.5 μM for L-tyrosine and 48.5 μM for L-DOPA as substrates. In this experiment, kojic acid was used as positive control. Kojic acid is used as an active component in cosmetic products distributed by Sansho Seiyaku Co., Ltd. (Ando *et al.*, 2010). With regards to quercetin glycoside compounds, Kubo and Kinst-Hori (1999) reported that quercetin glycosides such as isoquercitrin and rutin did not inhibit mushroom tyrosinase activity. The flavonoids reported by Fujii and Saito (2009) and Kubo and Kinst-Hori (1999) have a glycoside moiety in the C-3 position, but compound **1** has the glycoside moiety in the C-3' position. This difference should result in different activities concerning the melanin inhibition in B16 melanoma cells and tyrosinase inhibitory activity.

It should be noted that opposite results for an analogue of **1**, *i.e.* free quercetin, have been reported as follows: Quercetin enhanced melanogenesis in human melanoma cells and normal

Table II. Effect of quercetin-3'-*O*- β -D-glucoside on mushroom tyrosinase.

Compound	L-Tyrosine ^a	L-DOPA ^a
	IC_{50} [μM] ^b	IC_{50} [μM] ^b
Quercetin-3'- <i>O</i> - β -D-glucoside (1)	6.5	48.5
Kojic acid	5.3	14.0

^a Substrate.

^b The IC_{50} value was interpolated from graphed concentrations and determined graphically with statistical software.

Values show the mean \pm SD ($n = 3$).

epidermal melanocytes (Nagata *et al.*, 2004), and also the total melanin content in B16 melanoma cells (Kubo *et al.*, 2007). Recently, it was reported that quercetin suppressed melanin formation in B16 melanoma cells with decreased intracellular tyrosinase activity and protein expression (Fujii and Saito, 2009). The reason for the differences in the effect of quercetin on melanin production in cells remains unclear.

To our knowledge, this is the first report on compound **1** from the dried skin of *A. cepa* showing potential as a skin-whitening agent by inhibiting melanin biosynthesis in B16 melanoma cells and by inhibiting mushroom tyrosinase. Compound **1** is more potent than arbutin, the positive control. The inhibition of tyrosinase may play a part in decreasing the melanin formation in B16 melanoma cells. However, additional experiments are needed to determine the exact mechanism.

Antioxidant properties

In general, it is believed that compounds having antioxidant activity show antiaging, whitening, and anti-inflammatory activities (Choi *et al.*, 2008). One of the main mechanisms by which flavonoids exert their antioxidant activity consists in transferring electrons and/or H atoms to biological ROS (superoxide, hydroxyl, lipid alkoxy, and peroxy radicals) (Pietta, 2000). As mentioned above, compound **1** showed promising results as a whitening agent because it inhibited melanin formation in B16 melanoma cells. We conducted an antioxidant assay, ORAC, in order to determine its ability to counteract oxidative stress from UV

Table III. Effect of quercetin-3'-O- β -D-glucoside in the ORAC assay.

Compound	ORAC value (mean \pm SD) [μ mol TE/ μ mol]
Quercetin-3'-O- β -D-glucoside (1)	3.04 \pm 0.67
Quercetin	7.64 \pm 0.27

TE, trolox equivalent.

radiation. Table III shows the ORAC assay result of compound **1**, which was 3.04 μ mol TE/ μ mol. Quercetin as a positive control gave 7.64 μ mol TE/ μ mol. Quercetin is well known for its use as antioxidant, including in an ORAC assay as reported by Kohri *et al.* (2009), who obtained similar values to ours. Alluis and Dangles (2001) reported that this compound showed antioxidant activity in the DPPH assay, and that the presence of the glucosyl moiety in the C-3' position decreased the antioxidant activity and the apparent rate of H atom abstraction.

In summary, compound **1** from the dried skin of *A. cepa* is a promising compound that could be useful for treating hyperpigmentation, as a skin-whitening agent, and as an antioxidant. However, the safety of this compound is the primary consideration for its practical use by humans.

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