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; Kawai et al., 1999; Sanderson et al., 1999; Shutenko et al., 1999). Onion is one of the major sources of various biologically active phytomolecules, e.g., phenolic acids, flavonoids, cepaenes, thiosulfates, 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-methylpropionamidine) 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-d6 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 μl of 330 μM L-tyrosine or 200 μM L-di-hydroxy-phenylalanine (L-DOPA) solution were mixed with 600 μl of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C. Then, 33 μl of the sample solution and 33 μl 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 DOPAchrome, 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/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 μl of the standard (Trolox) or sample solution were mixed with 1.8 ml of 48 mM FL solution, and then incubated independently at 37 °C for 15 min. AAPH solution (900 μl, 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 μM 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

\[ AUC = 1 + \sum f_i / f_0, \]

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 μM). The regression of netAUC of Trolox on different concentrations was calculated as follows:

\[ Y = 0.4499X - 2.6812, \]

where \( X \) is the netAUX of Trolox and \( Y \) the Trolox concentration (μM).

The relative ORAC value was calculated by the following equation: relative ORAC value (μM TE/μmol) = (0.4499netAUCsample − 2.6812) / [sample concentration (μM)].

**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 · 10⁵ 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 μl of fresh medium, and 2 μl 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 · 10⁵ cells/well. After incubation, 50 μl 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-
Quercetin-3’-O-β-D-glucoside

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 I 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 I, melanin biosynthesis inhibitory activity. Some glycoside flavonoids, such as (+)-taxifolin-3-O-β-D-xylopyranoside, (+)-taxifolin-3-O-a-L-arabinofuranoside, quercetin-3-O-a-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 I. The data in Table II shows that compound I 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 Fuji and Saito (2009) and Kubo and Kinst-Hori (1999) have a glycoside moiety in the C-3 position, but compound I 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 I, i.e. free quercetin, have been reported as follows: Quercetin enhanced melanogenesis in human melanoma cells and normal

Table I. Effect of quercetin-3’-O-β-D-glucoside on melanin formation (IC$_{50}$) and cell viability in B16 melanoma cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ [μM]</th>
<th>Cell viability (% vs. control)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin-3’-O-β-D-glucoside (I)</td>
<td>38.8</td>
<td>92.0</td>
</tr>
<tr>
<td>Arbutin (positive control)</td>
<td>198.3</td>
<td>95.0</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>L-Tyrosinea</th>
<th>L-DOPAa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ [μM]$^b$</td>
<td>IC$_{50}$ [μM]$^b$</td>
</tr>
<tr>
<td>Quercetin-3’-O-β-D-glucoside (I)</td>
<td>6.5</td>
<td>48.5</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>5.3</td>
<td>14.0</td>
</tr>
</tbody>
</table>

$^a$ Substrate.

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

Values show the mean ± 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 \( \text{H} \) atoms to biological ROS (superoxide, hydroxyl, lipid alkoxy, and peroxyl 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 radiation. Table III shows the ORAC assay result of compound 1, which was 3.04 \( \mu \text{mol TE/µmol} \). Quercetin as a positive control gave 7.64 \( \mu \text{mol TE/µmol} \). 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 \( \text{H} \) 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.

**Acknowledgement**

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>ORAC value (mean ± SD) [µmol TE/µmol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin-3'-O-β-D-glucoside (1)</td>
<td>3.04 ± 0.67</td>
</tr>
<tr>
<td>Quercetin</td>
<td>7.64 ± 0.27</td>
</tr>
</tbody>
</table>

TE, trolox equivalent.


