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Anti-melanogenesis properties of quercetin- and its derivative-rich extract from *Allium cepa*

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ABSTRACT

In an effort to find a new whitening agent, we have found that the methanol extract of the dried skin of *Allium cepa* showed inhibition of melanin formation. Bioassay-guided fractionation led to the isolation of quercetin (**1**) and quercetin 4'-*O*-β-glucoside (**3**) from *A. cepa* as the inhibitors of melanin formation in B16 melanoma cells with IC_{50} values of 26.5 and 131 μM, respectively. In addition, we evaluated the effect of some quercetin derivatives, such as isoquercitrin (**2**), quercetin 3,4'-*O*-diglucoside (**4**), rutin (**5**) and hyperin (**6**) on B16 melanoma cells. These quercetin derivatives did not show any inhibition of melanin formation. Furthermore, the ORAC values of compounds **1**–**6** were 7.64, 8.65, 4.82, 4.32, 8.17 and 9.34 μmol trolox equivalents/μmol, respectively. Dried skin of red onion showed inhibitory activity against melanin formation in B16 melanoma cells, as well as antioxidant properties.

1. 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, Rodrígez-López, & García-Carmona, 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 or 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, hydrogen peroxide (H_2O_2) is generated, leading to the production of hydroxyl radicals ($HO^{\cdot}$) and other reactive oxygen species (ROS) (Perluigi et al., 2003).

The onion is a versatile vegetable that is consumed fresh, as well as in the form of processed products. The regular consumption of onions in food is associated with a reduced risk of neurodegenerative disorders, cancer, cataract, ulcer, osteoporosis, vascular disease and heart disease (Kaneko & Baba, 1999; Kawaii, Tomono, Katase, Ogawa, & Yano, 1999; Sanderson, Mclauchlin, & Williamson, 1999; Shutenko et al., 1999). Onion is one of the major sources of various biologically active phytomolecules, e.g., phenolic acids, flavonoids, cepaenes, thiosulfinates and anthocyanins (Singh et al., 2009). The major flavonoids found in the dry peel of the onion, which has usually been considered to be waste, contain large amounts of quercetin, quercetin glycoside and their oxidative products, which are effective antioxidants against the lethal effect of oxidative stress (Gulsen, Makris, & Kefalas, 2007; Prakash, Upadhyay, Singh, & Singh, 2007).

Onion (*Allium* species) plays an important role in traditional medicine in Indonesia; it is used as a diuretic, febrifuge and poultice, to cure wounds and to remove scars from the skin, and it suppresses the blood sugar level and platelet aggregation (de Padua, Bunyapraphatsara, & Lemmens, 1999).

Based on our preliminary screening data (not shown), the methanol extract of the dried skin of the red onion (*Allium cepa*) from Indonesia showed a potent melanin biosynthesis inhibitory activity on B16 melanoma cells. These findings led us to focus on the active compounds in the dried skin of the red onion.

2. Materials and methods

2.1. Chemicals

NaOH, DMSO, L-tyrosine, L-DOPA, and rutin (**5**) were purchased from Wako (Osaka, Japan). Mushroom tyrosinase, fluorescein

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sodium salt (FL), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or MTT, were obtained from Sigma (St. Louis, MO, USA). Quercetin-3,4'-*O*-diglucoside (**4**) and hyperin (**6**) were from Tokiwa Phytochemical (Tokiwa, Japan). EMEM was from Nissui Chemical Co. (Osaka, Japan). The ethylenediaminetetraacetic acid, or EDTA, was from Dojindo (Kumamoto, Japan). The 1,1-diphenyl-2-picrylhydrazyl (DPPH·) was from TCI (Tokyo, Japan) and isoquercitrin (**2**) was from Fluka (Steinheim, Germany). Other chemicals are of the highest grade commercially available.

2.2. 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 in the Wood Chemistry Laboratory, Department of Forest Product Technology, Faculty of Forestry, Mulawarman University, Indonesia.

2.3. Preparation of plant extracts

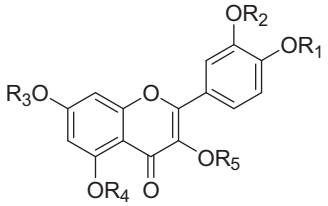
Plant materials (dried skin or flesh part of *A. cepa*) were dried at room temperature and powdered. The dried materials (17.38 g) were extracted with methanol (650 ml) at room temperature, with a shaker at 150 rpm over 48 h. The extract solutions were filtered and concentrated, *in vacuo*, to obtain the crude methanol extracts. The crude extracts were 1.75 g.

2.4. Isolation of quercetin (*1*) and quercetin 4'-*O*-β-glucoside (*3*)

The dried skin of the methanol extract of *A. cepa* (1.4 g) was subjected to silica gel column chromatography (71 g of Wakogel C-200, 3.5 × 50 cm). The column was 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) and 0:10 (100)] to give 33 fractions (Fr. 1–Fr. 33). The fractions Fr. 3 (66.9 mg) and Fr. 15 (84.7 mg) were analyzed by TLC (thin layer chromatography) and HPLC (high performance liquid chromatography). Based on NMR (nuclear magnetic resonance) analysis, Fr. 3 was identified as quercetin (**1**) and Fr. 15 as quercetin-4'-*O*-glucoside (**3**), by comparison of previous NMR data (Alfonso & Kapetanidis, 1994; Tanabe, Ogawa, Tesaki, & Watanabe, 1997), respectively (Fig. 1). The NMR spectra of compounds were recorded at 400 MHz on a JNM-AL400 FT NMR spectrometer (Jeo1). All compounds were dissolved in DMSO–d_6, or methanol–d_4, and chemical shifts were referred to deuterated solvents. The compounds were assigned for ^{1}H, ^{13}C, HMQC and HMBC.

2.5. HPLC analysis

All the crude extracts of dried skin and flesh of onion were dissolved in HPLC grade methanol, filtered through a sterile 0.22 μm Millipore filter and subjected to qualitative analysis by using a Waters 600 HPLC instrument. The instrument was equipped with a photodiode array (detector Waters 996), controller (Waters 600s), pump (Water 626), and auto sampler injector Model 231 (Gilson). ODS Inertsil C18 (4.6 mm i.d. × 250 mm) was used as a column. Data were integrated by Empower Build 1154-J series software (Waters). Separation was achieved by flow rate of 1 ml/min with methanol (60%)/water (40%) containing 1% trifluoroacetic acid, in an isocratic programme, by monitoring the absorbance at 250 nm.



1: $R_1 = R_2 = R_3 = R_4 = R_5 = H$
2: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = glc
3: R_1 = glc, $R_2 = R_3 = R_4 = R_5 = H$
4: R_1 = glc, $R_2 = R_3 = R_4 = H$, R_5 = glc
5: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = rha
6: $R_1 = R_2 = R_3 = R_4 = H$, R_5 = gal

Fig. 1. The structures of quercetin and its derivatives.

2.6. 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, Hampsch-Woodill, and Prior (2001) with some modification as follows: 300 μl of the standard (trolox) or sample solution was mixed with 1.8 ml of 48 nM FL solution, and then they were 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. Then, it was immediately placed in a fluorescence spectrophotometer (Model FP-6500, JASCO Co., Ltd., Tokyo, Japan), and measured every 5 s for 60 min at 37 °C (Ex: 485 nm, Em: 520 nm). A blank (FL + AAPH) using phosphate buffer, standard solutions (6.25–50 μM trolox), and sample solutions were all measured under the same conditions. Three independent assays were performed for each sample. The area under the fluorescence decay curve (AUC) was calculated as

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

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, as by Prior et al. (2003), using a equation $(Y)a + b(X)$ between trolox concentration (Y) (μM) and the net area under the FL decay curve (X). Linear regression was used in the range of 6.25–50 μM trolox. Data are expressed as micromoles of trolox equivalents (TE) per micromole of sample (μmol TE/μmol).

2.7. Cell culture

A mouse melanoma cell line, B16, was obtained from the RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml of theophylline. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.8. Inhibitory effect of melanin biosynthesis using cultured B16 melanoma cells

This assay was done as described by Arung, Shimizu, and Kondo (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 plates of 24-well plastic culture plates (1 plate for determining of

melanin and the other for cell viability) at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 μl of fresh media 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 then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed (see below).

2.9. 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 cell pellet was dissolved in 1.0 ml of 1 N NaOH. The crude cell extracts were assayed using a microplate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture.

2.10. Cell viability

Cell viability was determined by use of the micro-culture tetrazolium technique (MTT). The MTT assay provides 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 1×10^5 cells/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)] were 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 0.04 N HCl) was pipetted into the plate, and the absorbance was measured at 570 nm relative to 630 nm.

3. Results and discussion

3.1. The effect of the methanol extract of the dried skin and flesh of A. cepa on B16 melanoma

The diameter of Indonesian red onion (*A. cepa*) is approximately 1.5–2.0 cm. The thickness of the dried skin is approximately 0.5–1.0 mm. Figs. 2 and 3 show the effects of the methanol extract of the dried skin and flesh of *A. cepa* on B16 melanoma cells, respectively. The dried skin extract dose-dependently inhibited melanin formation in B16 melanoma cells. The inhibition of 40–50% of melanin formation was evident at concentrations of 50 and 100 μg/ml without any cytotoxicity. Arbutin, which is used in skin-whitening cosmetics, was used as a positive control (Virador, Kobayashi, Matsunaga, & Hearing, 1999). In contrast, the extract of the flesh of the onion did not lead to melanin inhibition, even at concentrations up to 250 and 500 μg/ml. According to these data, the dried skin extract is more potent than is the flesh extract. This result may be related to the amount of active compound in the dried skin, as shown in Figs. 4 and 5. Fig. 4 depicts HPLC chromatograms obtained by the injection of 20 μl of solution which contained methanol extract of the dried skin of *A. cepa* at a concentration of 1 mg/ml. Based on the methanol extract of the dried skin, the contents of quercetin, isoquercitrin; quercetin 4'-*O*-glucoside; and quercetin 3,4'-*O*-diglucoside were 13.8%, 10.3%, 6.4% and 8.3%, respectively. We tried to analyze the flesh extract at a concentration of 1 mg/ml but each peak of the compounds was too small to be detected; therefore, we increased the concentration for HPLC analysis. Fig. 5 shows the HPLC chromatogram obtained by the injection of 20 μl of solution which contained methanol extract of the flesh of *A. cepa*

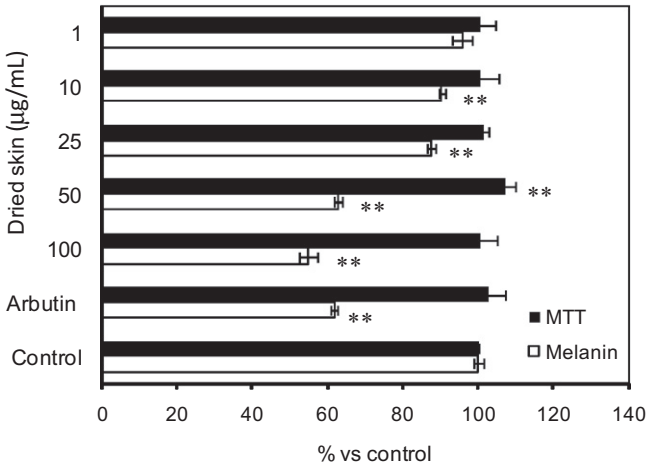


Fig. 2. Effect of the methanol extracts of the dried skin of *A. cepa* on B16 melanoma cells [Arbutin: 100 μg/ml]. Each column represents the mean ± SD of three independent tests (Student's *t*-test). Significantly different from the control value: $P < 0.01$ (**).

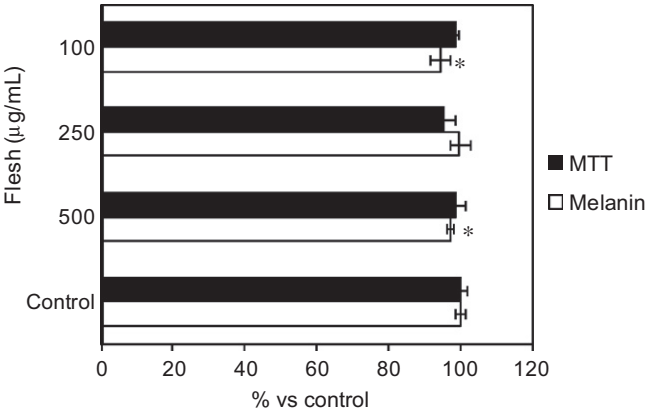


Fig. 3. Effect of the methanol extracts of the flesh of *A. cepa* on B16 melanoma cells. Each column represents the mean ± SD of three independent tests (Student's *t*-test). Significantly different from the control value: $P < 0.05$ (*).

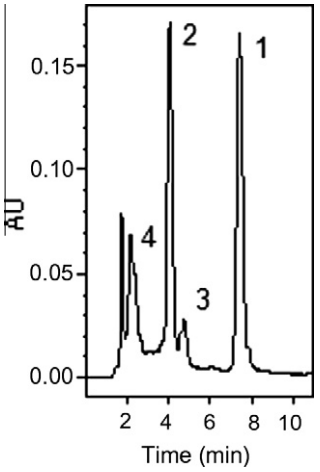


Fig. 4. HPLC analysis of the methanol extracts of dried skin of *A. cepa* [1. Quercetin, 2. isoquercitrin, 3. quercetin 4'-*O*-glucoside, 4. Quercetin 3,4'-*O*-diglucoside, flow rate: 1 ml/min of MeOH (60): water (40); sample concentration: 1 mg/ml, wavelength 250 nm, injection volume: 20 μl].

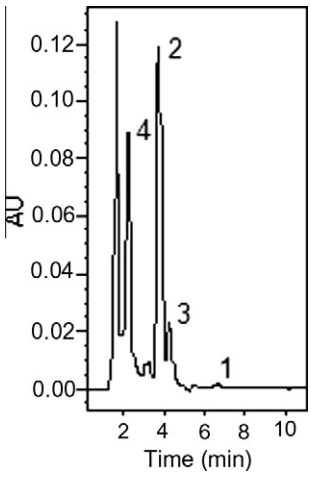


Fig. 5. HPLC analysis of the methanol extract of flesh of *A. cepa* [1. Quercetin, 2. Isoquercitrin, 3. Quercetin 4'-*O*-glucoside, 4. Quercetin 3,4'-*O*-diglucoside, flow rate: 1 ml/min of MeOH (60): water (40); sample concentration : 11 mg/ml, wavelength 250 nm, injection volume: 20 μl].

at a concentration of 11 mg/ml. Based on the methanol extract of the flesh, the contents of quercetin, isoquercitrin, quercetin 4'-*O*-glucoside and quercetin 3,4'-*O*-diglucoside were 0.01%, 0.59%, 0.35% and 0.50%, respectively. As seen on the *Y* axis of both figures, the content of quercetin derivatives in the methanol extract of the dried skin of *A. cepa* was apparently much higher than was that in the extract of the flesh. Also, it was shown that the extract of dried skin contained quercetin (**1**) and isoquercitrin (**2**) dominantly, but in the flesh extract, isoquercitrin (**2**) and quercetin 3,4'-*O*-diglucoside (**4**) were also abundant. It is reported that the contents of quercetin, quercetin 4'-*O*-glucoside and quercetin 3,4'-*O*-diglucoside in different parts of the onion bulb (*A. cepa* L.) were different as they also were in the various cultivars (Beesk et al., 2010). The outer layer of *A. cepa* had approximately 2.2%, 2.7%, 1.7% of quercetin, quercetin 4'-*O*-glucoside, and quercetin 3,4'-*O*-diglucoside (per dry weight of outer layer), respectively. In the inner parts, the contents of quercetin, quercetin 4'-*O*-glucoside, and quercetin 3,4'-*O*-diglucoside were 0%, 0.53% and 0.72%, respectively. In our opinion, the difference of quercetin and its derivative contents should affect the difference in their melanin inhibitory activity, as depicted in Figs. 2 and 3.

3.2. Isolated compounds

The biologically-guided fractionation of methanol extracts of *A. cepa* on B16 melanoma cells led us to focus on the active fraction of the dried skin extract. As a result of fractionation, Fr. 3 was identified as one of the active compounds as well as Fr. 15. Thus, NMR assignment was performed to elucidate the structure of these fractions by comparison with previous data (Alfonso & Kapetanidis, 1994; Tanabe et al., 1997). It was revealed that Fr. 3 and Fr. 15 were **1** (69.9 mg, 4.9% yield in the extract) and **3** (84.7 mg, 6.1% yield in the extract), respectively.

3.3. Anti-melanogenesis properties

The anti-melanogenesis effects of isolated compounds **1** and **3** (Fig. 1) from the dried skin of *A. cepa* were determined by using B16 melanoma cells. Table 1 depicts the IC_{50} values for the melanin biosynthesis inhibition of quercetin and its derivates. The activities of the tested compounds, in terms of IC_{50} values, decreased in the following order: **1** (<50 μM), **3** (100–150 μM), **4**, **5**, **2** and **6** (>150 μM). Notably, isolated compounds **1** and **3** from *A. cepa*,

Table 1
Effect of quercetin and its derivatives on B16 melanoma cells ($n = 3$).

Compounds	Melanin inhibition IC_{50} (μM)	Cell viability (% vs control)[a]
Quercetin (**1**)	26.5	88
Quercetin-3-*O*-glucoside (Isoquercitrin) (**2**)	>215	107 at >215 μM
Quercetin-4'-*O*-glucoside (**3**)	130.6	82
Quercetin-3,4'-*O*-diglucoside (**4**)	>159	110 at >159 μM
Quercetin-3-*O*-rhamnoside (rutin) (**5**)	>163	101 at >163 μM
Quercetin-3-*O*-galactoside (hyperin) (**6**)	>215	90 at >215 μM
Arbutin (positive control)	198.3	95

[a] Cell viability (%) at the concentration of IC_{50} for melanin formation on B16 melanoma cells.

were found to be more-potent inhibitors of melanin formation in B16 melanoma cells than was the positive control, arbutin (198 μM). It should be noted that the opposite results were reported previously, namely that **1** enhanced melanogenesis in human melanoma cells and normal epidermal melanocyte (Nagata, Susumu, Takeyama, Homma, & Osamura, 2004). Kubo, Nitoda, and Nihei (2007) also reported that **1** enhanced the total melanin content in B16 melanoma cells. Recently, it was reported that **1** suppressed melanin formation in B16 melanoma cells with decreased intracellular tyrosinase activity and its protein expression (Fujii & Saito, 2009). The reason for the difference of the effect of **1** on melanin production in cells remains unclear.

Based on our analysis, the IC_{50} of **1** to inhibit melanin formation was 26.5 μM, with 88% cell viability, while that of **3** was 131 μM, with 82% cell viability. The attachment of a glucoside moiety in quercetin tended to reduce the ability of quercetin to inhibit melanin formation in B16 melanoma cells. These results led us to evaluate other quercetin derivatives, such as **2**, **4**, **5**, and **6** (Fig. 1) in B16 melanoma cells. Compounds **2**, **5** and **6,** which represent different glycosyl moieties (glucoside, rhamnoside and galactoside) at C-3, displayed no inhibition of melanin formation. Concerning this result, a previous report showed that quercetin 3-*O*-L-arabinofuranoside did not suppress melanin formation (Fujii & Saito, 2009). These results revealed that the attachment of a glycosyl moiety at the C-3 position may decrease the ability of quercetin to reduce the melanin formation. We concluded that the hydroxyl group at the C-3 position is essential to the inhibition of melanin formation in B16 melanoma cells. To our knowledge, this is the first report showing that the dried skin of *A. cepa* and its isolated compound, **3,** showed the ability to act as a potent skin-whitening agent by inhibiting the melanin formation in B16 melanoma cells, similarly to arbutin, which was used as a positive control. Therefore, further experiments are needed to determine the exact mechanism of this compound.

3.4. Antioxidant properties

Skin is a major candidate for and target of oxidative stress caused by reactive species (RS), including reactive oxygen species and reactive nitrogen species. RS are major and significant contributors to skin hyperpigmentation and skin aging (Kim, Kang, & Yokozawa, 2008). It has been generally believed that agents having antioxidant activity show anti-aging, whitening, and anti-inflammatory activities (Choi, Song, Hur, & Sim, 2008). Since compounds **1** and **3** have shown promising results as whitening agents using B16 melanoma cells, we conducted an antioxidant assay (ORAC) in order to determine their ability to counteract oxidative stress from UV radiation. Table 2 shows the ORAC value results of compounds **1**–**6**, which were 7.64, 8.65, 4.82, 4.32, 8.17 and 9.34 μmol TE/μmol, respectively. Compounds **1**, **2**, **5** and **6** showed

Table 2
Effect of quercetin and its derivatives on ORAC assay ($n = 3$).

Compounds	ORAC values (mean ± SD) (μmol TE/μmol)
Quercetin (**1**)	7.64 ± 0.27
Quercetin-3-*O*-glucoside (Isoquercitrin) (**2**)	8.65 ± 0.36
Quercetin-4'-*O*-glucoside (**3**)	4.82 ± 0.67
Quercetin-3,4'-*O*-diglucoside (**4**)	4.32 ± 0.25
Quercetin-3-*O*-rhamnoside (rutin) (**5**)	8.17 ± 0.41
Quercetin-3-*O*-galactoside (hyperin) (**6**)	9.34 ± 0.24

TE, trolox equivalent.

more potent antioxidant activity than did **3** and **4**. In the ORAC assay, quercetin showed an ORAC value similar to that reported by Kohri et al. (2009). However, we did not find any previous report of an ORAC assay for **3**. Based on the results in Table 1, it was revealed that 4'-glycosylation of quercetin decreased its ability as an antioxidant, as represented by ORAC values.

The potencies of **1** and **3** as antioxidants might be related to the presence of hydroxyl groups in the form of a catechol moiety, as reported by Cao, Sofic, and Prior (1997). Moreover, the relationship between flavonoid structure and antioxidant activity has been studied, and it has been found that 3',4'-dihydroxy substitution in the B ring, as in quercetin, increases the antioxidant activity substantially compared with a mono-hydroxy substituent, as in kaempferol (Cotelle et al., 1996; Rice-Evans, Miller, Bolwell, Branley, & Pridham, 1995).

4. Conclusion

Our study demonstrated that the melanin inhibition ability of quercetin derivatives with glycosyl moieties at C-3 is reduced. Also, our results showed that quercetin derivatives with a glycosyl moiety at C-4' had reduced antioxidant activity. In addition, compounds **1** and **3,** from the dried skin of *A. cepa*, are promising compounds that could be useful for treating hyperpigmentation as skin-whitening agents and as antioxidants. However, it should be noted that safety is a primary consideration before practical use in humans.

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