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Reassessment of antioxidant activity of arbutin: Multifaceted evaluation using five antioxidant assay systems

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Abstract
Arbutin, a practically used skin-lightening agent, has been reported to possess a weak antioxidant activity compared to that of its precursor, hydroquinone. However, its antioxidant activity has not been systematically evaluated. Hence, this study reassessed its activity using five assay systems. Assays were first performed using model radicals, DPPH radical and $ABTS^{\bullet+}$. Arbutin showed weak DPPH radical-scavenging activity compared to that of hydroquinone, but showed strong $ABTS^{\bullet+}$-scavenging activity. Its activity by ORAC assay was then evaluated using a physiologically relevant peroxyl radical. Arbutin exerted weak but long-lasting radical-scavenging activity and showed totally the same antioxidant activity as that of hydroquinone. Finally, it was shown that, in two cell-based antioxidant assays using erythrocytes and skin fibroblasts, arbutin exerted strong antioxidant activity comparable or even superior to that of hydroquinone. These findings indicate that the antioxidant activity of arbutin may have been under-estimated and suggest that it acts as a potent antioxidant in the skin.

Keywords: *Arbutin, hydroquinone,* in vitro *antioxidant assay, cell-based antioxidant assay, structure–activity relationship, radical-scavenging activity*

Introduction

The association of reactive oxygen species (ROS) and free radicals with many disease states is now well recognized and antioxidants have attracted considerable attention [1,2]. There have been many studies on natural and synthetic antioxidants from various viewpoints and a structure–activity relationship (SAR) in radical-scavenging activities using unnatural model radicals has been extensively studied [3–10]. Classical SAR studies are associated with the number of oxidizable -OH groups in a molecule [4,5,8,9]. For instance, it is well known that ascorbic acid (AA) has two oxidizable -OH groups at C-2 and C-3 positions (Figure 1), so that one molecule of it can scavenge two molecules of radicals, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [4] and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\bullet+}$) [11]. Thus, the more oxidizable -OH moieties are present in the molecule, the more quantities of radicals are likely to be quenched. 2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) and 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG) are 2-*O*-substituted AA derivatives in which the oxidizable -OH group at the C-2 position of AA is replaced with the glucosyl group (Figure 1). According to classical SAR theory, these derivatives should diminish radical-scavenging activities. However, we showed that, although they reacted to DPPH radical and $ABTS^{\bullet+}$ more slowly than AA, they could quench more radicals than AA in a long-term reaction (hour-time-scale) under

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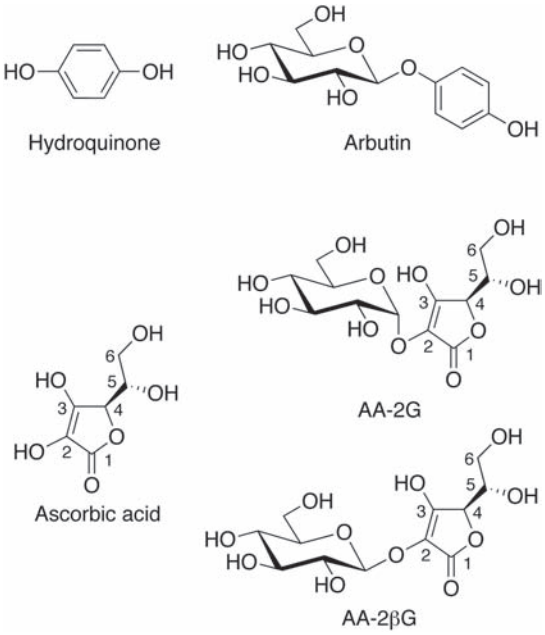

Figure 1. Chemical structures of hydroquinone, arbutin, AA, AA-2G and AA-2βG.

optimal conditions [12,13]. Furthermore, these AA derivatives showed antioxidant activities comparable to that of AA in a more biologically relevant *in vitro* antioxidant assay system, oxygen radical absorbance capacity (ORAC) assay [13] and in a cell-based antioxidant assay system, oxidative haemolysis inhibition assay (OxHLIA) [13–15]. Hence the radical-scavenging properties of the glucosides of AA are largely altered from that of AA, but their comprehensive antioxidant activities may be retained.

Hydroquinone is a potent antioxidant with 2 oxidizable -OH groups in the molecule as well as AA (Figure 1). A glucoside of hydroquinone, arbutin, retains one oxidizable -OH group of hydroquinone similar to AA-2G and AA-2βG (Figure 1), so that it appears to act as an antioxidant. In fact, there have been some studies showing that arbutin exhibited antioxidant activity but that it was not strong compared to that of hydroquinone [16–18]. However, in those studies, the activity of arbutin was assessed in relatively short-term (minute-time scale) [17,18] or mainly from the viewpoint of kinetics [16]. Considering the case of AA-2G and AA-2βG, arbutin may show its characteristic of radical-scavenging in a long-lasting manner and therefore its antioxidant activity may have been under-estimated.

Arbutin is well known as an inhibitor of tyrosinase, which is a key enzyme involved in melanin synthesis and it is widely used as a skin-lightening agent for cosmetic products [19]. In addition to this, we show here that arbutin possesses a long-lasting radical-scavenging property by DPPH radical-scavenging assay and $ABTS^{\bullet+}$-scavenging assay and that the antioxidant activity of arbutin was comparable to or even superior to that of hydroquinone in the ORAC assay and two cell-based antioxidant assays using erythrocytes and human skin fibroblasts. Antioxidants have an important role in the skin to prevent skin ageing, skin disorders and skin diseases [20]. Hence, the results obtained here suggest that arbutin acts not only as a skin-lightening agent by inhibiting tyrosinase activity but also as a useful antioxidant in the skin.

Materials and methods

Chemicals

Arbutin and hydroquinone were purchased from Nacalai Tesque Inc. (Kyoto, Japan). AA, sodium fluorescein and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). DPPH was from Aldrich Chemical (Milwaukee, WI). ABTS, H_2O_2 (3%) and horseradish peroxidase (HRP; type VI-A, essentially a salt-free 1310 units/mg solid) were from Sigma Chemical (St. Louis, MO). Sheep erythrocytes were from Nippon Bio-Supp. Center (Tokyo, Japan). Reagents were used without further purification. All water used was Milli-Q grade.

DPPH radical-scavenging assay

The DPPH radical-scavenging activities were assessed as described in a previous paper [12]. Briefly, DPPH radical (100 μM) was mixed with an antioxidant (20 μM) in 60% ethanol/40% citric acid sodium citrate buffer (10 mM, pH 5). The reaction was carried out under an atmosphere of argon at 25°C for 2 h. The decrease of DPPH radical concentration was monitored by measuring the absorbance at 524 nm with a spectrophotometer (Shimadzu UV-1200, Kyoto, Japan).

$ABTS^{\bullet+}$-scavenging assay

The $ABTS^{\bullet+}$-scavenging activities were assessed as described in a previous paper [21]. Briefly, $ABTS^{\bullet+}$ (100 μM) generated with an $ABTS/H_2O_2$/HRP system was mixed with an antioxidant (20 μM) in citric acid-sodium citrate buffer (50 mM, pH 5). The reaction was carried out under an atmosphere of argon at 25°C for 2 h. The decrease of $ABTS^{\bullet+}$ concentration was monitored by measuring the absorbance at 730 nm with a spectrophotometer.

ORAC assay

The ORAC assay was carried out as described in a previous paper [13]. Briefly, fluorescein (60 nM), antioxidant (3 μM) and AAPH (18.75 mM) were incubated in 200 μl of KH_2PO_4-K_2HPO_4 buffer

(75 mM, pH 7.0) at 37°C in a 96-well plate. The fluorescence (Ex: 485 nm, Em: 520 nm) was monitored every 2 min for 90 min by Powerscan HT (DS Pharma Biomedical, Osaka, Japan).

OxHLIA

The OxHLIA was carried out as described in previous papers [13,15]. Briefly, sheep erythrocytes suspended at a concentration of 0.7% (v/v) in phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na_2HPO_4 and 1.9 mM NaH_2PO_4, pH 7.4) were incubated with 40 mM of AAPH in the presence of an antioxidant (50 μM) at 37°C with shaking. The degree of haemolysis (%) was monitored every 15 min for 3 h from the concentration of haemoglobin in the centrifuged supernatant by measuring the absorbance at 524 nm.

Oxidative cell death protection assay

Normal human skin fibroblasts (NB1RGB; RIKEN Cell Bank, Tsukuba, Japan) were cultured with Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were suspended in medium at an initial density of 1×10^5 cells/ml. Each 100 μl of cell suspension was seeded in a 96-well culture plate (NUNC, Roskilde, Denmark). After 24-h incubation, 50 μl of arbutin (62.5–1000 μM, final concentration) or hydroquinone (62.5 and 125 μM, final concentration) containing medium and 50 μl of 20 mM (final concentration) AAPH was added. Skin fibroblasts were cultured at 37°C for 24 h, washed three times with PBS and labelled with 100 μl of 5 μM calcein-AM (Dojindo, Kumamoto, Japan) in PBS. After 30 min with calcein-AM, the cells were lysed with addition of 20 μl of Triton X-100 (0.6%, v/v) in PBS. The fluorescence from calcein-positive viable cells was measured with excitation at 485 nm and emission at 527 nm using with Fluoroskan Ascent (Labosystems, Helsinki, Finland).

Results

DPPH radical-scavenging assay and ABTS$^{\bullet+}$-scavenging assay

DPPH radical and ABTS$^{\bullet+}$ are relatively stable radicals. Their characteristic colours disappear when they are quenched and so the decrease of these radicals can be easily monitored by a spectrometer [22]. Because pH of the skin surface is ~5 [23], we assessed the DPPH radical- and ABTS$^{\bullet+}$-scavenging activities of arbutin in buffered solutions at pH 5 and compared them with those of hydroquinone and AA (Figure 2). AA was used as a reference. Hydroquinone and AA showed nearly the same reaction profile in both assays, i.e. 20 μM of these antioxidants rapidly quenched ~40 μM of DPPH radical and ABTS$^{\bullet+}$ within 1 or 5 min. Thus, their reaction stoichiometries (number of radical molecules reduced by one molecule of antioxidant) were ~2 and this was consistent with a previous report [24] and the theory of the classical SAR. On the other hand, arbutin slowly and continuously reacted with DPPH radical as expected (Figure 2A). The stoichiometry of this reaction was 0.7 at the time point of 2 h, but this value might be slightly increased afterward because it seems that the reaction had not

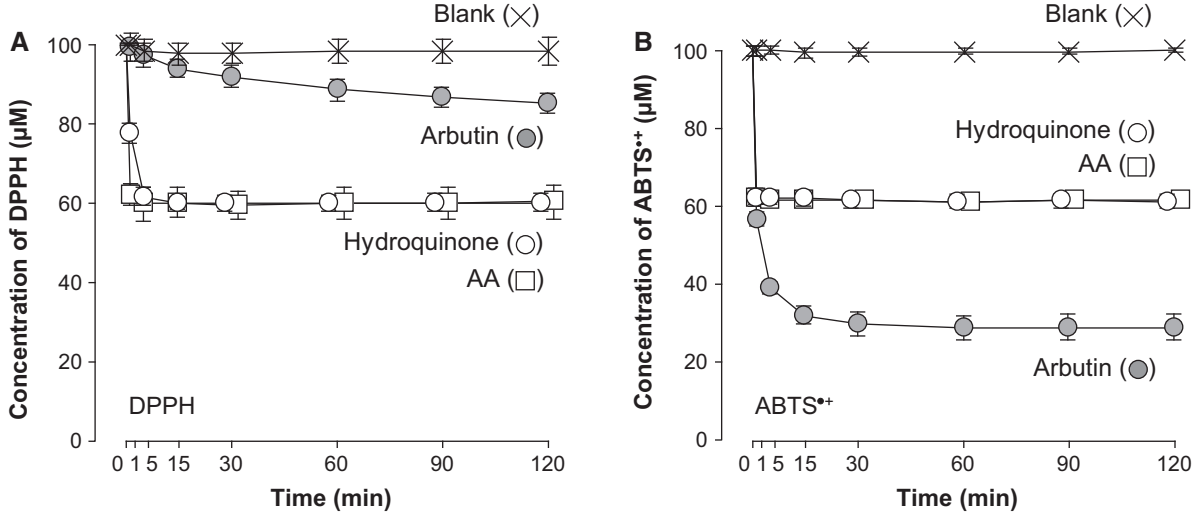

Figure 2. Time courses of DPPH radical- (A) and ABTS$^{\bullet+}$-scavenging (B) reaction of arbutin, hydroquinone and AA. Arbutin, hydroquinone or AA (20 μM) and DPPH radical or ABTS$^{\bullet+}$ (100 μM) were incubated at 25°C in 60% ethanol/40% citrate buffer (10 mM, pH 5) or citrate buffer (50 mM, pH 5), respectively. Changes in the remaining radicals were measured at the indicated times. Each value is the mean ± SD of three separate experiments. Absence of SD bar means that the SD bar is within the symbol.

yet been completed. In contrast, the amount of $ABTS^{\bullet+}$ scavenged by arbutin in the first minute was comparable to that scavenged by hydroquinone and AA (Figure 2B). Interestingly, the reaction between arbutin and $ABTS^{\bullet+}$ further proceeded until 30 min and gave high reaction stoichiometry such as 3.5. Arbutin showed weak activity in the DPPH radical-scavenging assay but unexpectedly strong activity in the $ABTS^{\bullet+}$-scavenging assay. This discrepancy led us to assess the antioxidant efficacy of arbutin in more physiologically relevant assay systems, because DPPH radical and $ABTS^{\bullet+}$ are both unnatural radical species that do not exist in the human body.

ORAC assay

The ORAC assay utilizes an AAPH-derived peroxyl radical, which mimics lipid peroxyl radicals involved in lipid peroxidation chain reaction *in vivo*. Inhibition of peroxyl-radical induced oxidations of a fluorescent probe, fluorescein, by antioxidants is serially monitored [22]. Because fluorescein diminishes fluorescence under acidic conditions, we performed the ORAC assay at pH 7, conventional pH for this assay [25]. Although it is different from the pH of skin (pH 5) [23], Prior et al. [22] reported that AAPH-derived peroxyl radical-scavenging reactions are pH-independent. In the case of hydroquinone and AA the fluorescence was completely retained in the early phase and decayed rapidly after lag times of 25 and 10 min, respectively (Figure 3). On the other hand, arbutin failed to suppress the fluorescent decay completely but continued partial inhibition for a longer period than hydroquinone and AA. As a result, the remaining fluorescence intensity of arbutin was more than that of hydroqunone and AA after 30 min. In the ORAC assay, the total extent of antioxidant activity is represented by the net area under the curve (net AUC), which is

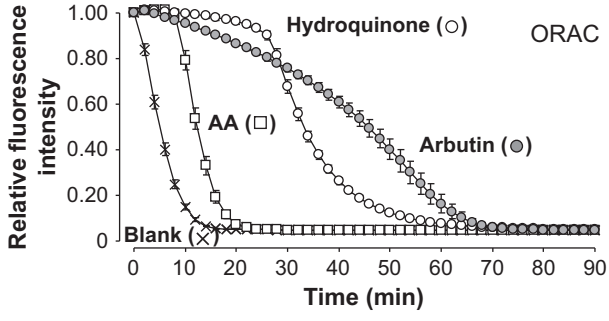

Figure 3. ORAC assay for arbutin, hydroquinone and AA. The reaction mixtures containing arbutin, hydroquinone or AA (3.0 μM), fluorescein (60 nM) and AAPH (18.75 mM) in 200 μl of KH_2PO_4-K_2HPO_4 buffer (75 mM, pH 7.0) were incubated at 37°C for 90 min. Changes in fluorescence intensity of fluorescein was monitored. Each value is the mean ± SD of triplicate experiments. Absence of SD bar means that the SD bar is within the symbol.

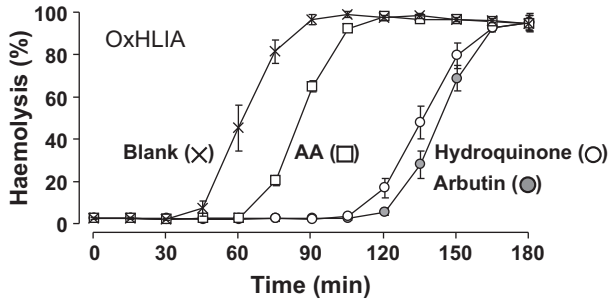

Figure 4. OxHLIA for arbutin, hydroquinone and AA. Sheep erythrocytes at 0.7% (v/v) suspension in PBS were incubated with 40 mM of AAPH in the presence of 50 μM of arbutin, hydroquinone or AA at 37°C for 180 min with shaking. Each value is the mean ± SD of three separate experiments. Absence of SD bar means that the SD bar is within the symbol.

obtained by subtracting the area under the blank curve from the area under the sample curve [25]. The net AUC was in the order of arbutin ≈ hydroquinone > AA. Thus, arbutin showed potent activity comparable to that of hydroquinone in the ORAC assay and we therefore confirmed its antioxidant activity in a cell-based assay.

Oxidative haemolysis inhibition assay (OxHLIA) and oxidative cell death protection assay

OxHLIA is a cell-based antioxidant assay using the same radical source as that used for the ORAC assay [15,26]. The oxidation of erythrocyte membranes by AAPH-derived peroxyl radical induces the oxidation of lipids and proteins and eventually causes haemolysis and this haemolysis was inhibited by each antioxidant (Figure 4). The order of inhibition was arbutin ≥ hydroquinone > AA, which was similar to that observed in the ORAC assay (Figure 3).

Finally, protective effects of arbutin and hydroquinone against AAPH-induced cell death on human skin fibroblasts were investigated (Figure 5). Arbutin significantly showed the protective effects against oxidative stress in fibroblasts, but hydroquinone did not. Hydroquinone had cytotoxicity at the concentration of 125 μM and above even in the absence of AAPH (data not shown), suggesting that the inherent antioxidant activity of hydroquinone was interfered with by its cytotoxic property. On the other hand, arbutin showed no influence on the cell viability in the absence of AAPH at the all concentrations tested (data not shown). As a result, the antioxidant effect of arbutin was superior to that of hydroquinone in the oxidative cell death protection assay.

Discussion

Hydroquinone is a potent antioxidative compound and its monoglucoside, arbutin, was thought to

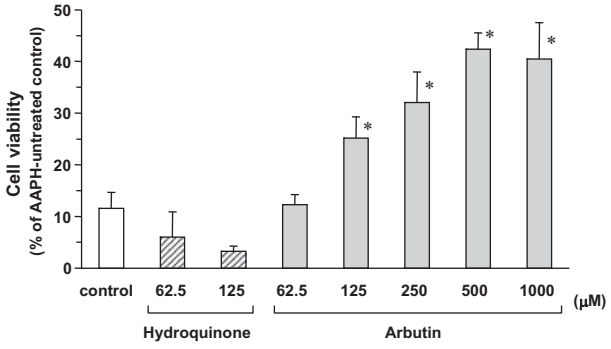

Figure 5. Protective effects of arbutin and hydroquinone against AAPH-induced toxicity on human skin fibroblasts. Human skin fibroblasts were cultured with 20 mM of AAPH in the presence of arbutin and hydroquinone at 37°C for 24 h. Each value is the mean ± SD of three separate experiments. The statistical significance of differences was established by one-way analysis of variance and Dunnet's test. Asterisk represents a significant difference from control at $p < 0.01$.

decrease antioxidant activity according to the classical SAR theory. In fact, the DPPH radical-scavenging activity of arbutin was diminis`hed compared to that of hydroquinone both in reaction rate and reaction stoichiometry (Figure 2A), as shown in previous studies [17,18]. However, arbutin showed strong $ABTS^{\bullet+}$-scavenging activity, especially in reaction stoichiometry (Figure 2B). The reaction stoichiometry, 3.5, was superior to the number of oxidizable -OH groups in the molecule and even to the reaction stoichiometry of its precursor, hydroquinone. The reaction between arbutin and $ABTS^{\bullet+}$ may proceed via complicated reactions followed by a simple electron transfer reaction. In any event, arbutin had a long-lasting radical-scavenging property, which is greatly different from the radical-scavenging property of hydroquinone (Figure 2). As several researchers pointed out, it is important to assess antioxidant efficacy by several methods [22,27], especially when different results are obtained from two assays. We therefore performed the ORAC assay, which is based on inhibition of the biologically relevant peroxyl radical-induced oxidation of fluorescein (Figure 3). Unlike hydroquinone, arbutin could not fully inhibit the oxidation of fluorescein. This indicated that the reactivity of arbutin against AAPH-derived peroxyl radical was lower than that of hydroquinone. On the other hand, the duration of the partial inhibition by arbutin was longer than that by hydroquinone. As a result, arbutin showed totally the same antioxidant activity as hydroquinone in the ORAC assay. Ioku et al. [16] reported a similar tendency in the case of inhibition of AAPH-induced methyl linoleate peroxidation; that is, arbutin showed lower reactivity against AAPH-derived peroxyl radical but a longer inhibition period than hydroquinone, although they did not particularly emphasize it. The ORAC assay uses biologically relevant peroxyl radicals, but the oxidizable target for peroxyl radicals, fluorescein, is not a mimic of a certain biomolecule. Therefore, we confirmed that arbutin exerted potent antioxidant activity comparable or even superior to that of hydroquinone by two cell-based antioxidant assays using erythrocytes (Figure 4) and human skin fibroblasts (Figure 5). Taken together, these results suggest that although arbutin had a different radical-scavenging property from that of hydroquinone, its total antioxidant activity may be equal to that of hydroquinone at least *in vitro*.

As mentioned in the Introduction, arbutin has been practically used as a skin-lightening cosmetic ingredient owing to its potent inhibitory effect on melanin synthesis. On the other hand, its aglycone, hydroquinone, has a stronger skin-whitening effect than that of arbutin [28] and is used not only as a cosmetic ingredient but also as a therapeutic agent for melasma and disorders of hyperpigmentation [29]. Recently, Bang et al. [18] showed that normal human skin microflora could hydrolyse arbutin to hydroquinone and suggested that the skin-lightening and antioxidant effects of arbutin were enhanced by hydroquinone formation *in vivo* [18]. However, they assessed antioxidant activity only by the DPPH assay. Arbutin is certainly less effective in skin lightening than hydroquinone, but the comprehensive antioxidant efficacy of arbutin may be comparable to that of hydroquinone based on the data presented here. In addition, it should be noted that hydroquinone showed cytotoxicity against normal human skin fibroblasts (Figure5) and its long-term safety has been controversial [30], while arbutin has been shown to have much less toxicity than that of hydroquinone [28]. Therefore, the antioxidant activity of arbutin *per se* could be more helpful in daily skin care than previously recognized [16–18].

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References

[1] Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. Nutrition 2002;18:872–879.
[2] Seifried HE, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. J Nutr Biochem 2007;18:567–579.

[3] Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958;181:1199–1200.
[4] Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebenson Wiss Technol 1995;28:25–30.
[5] Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–956.
[6] Ancerewicz J, Migliavacca E, Carrupt PA, Testa B, Brée F, Zini R, Tillement JP, Labidalle S, Guyot D, Chauvet-Monges AM, Crevat A, Le Ridant A. Structure-property relationships of trimetazidine derivatives and model compounds as potential antioxidants. Free Radic Biol Med 1998;25:113–120.
[7] Yokozawa T, Chen CP, Dong E, Tanaka T, Nonaka GI, Nishioka I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2 picrylhydrazyl radical. Biochem Pharmacol 1998;56:213–222.
[8] Senba Y, Nishishita T, Saito K, Yoshioka H, Yoshioka H. Stopped-flow and spectrophotometric study on radical scavenging by tea catechins and the model compounds. Chem Pharm Bull 1999;47:1369–1374.
[9] van den Berg R, Haenen GRMM, van den Berg H, van der Vijgh W, Bast A. The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay. Food Chem 2000; 70:391–395.
[10] Sekher Pannala A, Chan TS, O'Brien PJ, Rice-Evans CA. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. Biochem Biophys Res Commun 2001;282: 1161–1168.
[11] Arnao MB, Cano A, Hernández-Ruiz J, García-Cánovas F, Acosta M. Inhibition by L-ascorbic acid and other antioxidants of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods. Anal Biochem 1996;236:255–261.
[12] Takebayashi J, Tai A, Gohda E, Yamamoto I. Characterization of the radical-scavenging reaction of 2-*O*-substituted ascorbic acid derivatives, AA-2G, AA-2P, and AA-2S: a kinetic and stoichiometric study. Biol Pharm Bull 2006;29:766–771.
[13] Takebayashi J, Yagi Y, Ishii R, Abe S, Yamada K, Tai A. Antioxidant properties of 2-*O*-β-D-glucopyranosyl-L-ascorbic acid. Biosci Biotechnol Biochem 2008;72:1558–1563.
[14] Takebayashi J, Kaji H, Ichiyama K, Makino K, Gohda E, Yamamoto I, Tai A. Inhibition of free radical-induced erythrocyte hemolysis by 2-*O*-substituted ascorbic acid derivatives. Free Radic Biol Med 2007;43:1156–1164.
[15] Takebayashi J, Chen J, Tai A. A method for evaluation of antioxidant activity based on inhibition of free radical-induced erythrocyte hemolysis. In: Armstrong D, editor. Advanced protocols in oxidative stress II, series: Methods in molecular biology, 594. Clifton: Humana Press; 2010. 287–296.
[16] Ioku K, Terao J, Nakatani N. Antioxidative activity of arbutin in a solution and liposomal suspension. Biosci Biotechnol Biochem 1992;56:1658–1659.
[17] Hisatomi E, Matsui M, Kobayashi A, Kubota K. Antioxidative activity in the pericarp and seed of Japanese pepper (*Xanthoxylum piperitum* DC). J Agric Food Chem 2000;48: 4924–4928.
[18] Bang SH, Han SJ, Kim DH. Hydrolysis of arbutin to hydroquinone by human skin bacteria and its effect on antioxidant activity. J Cosmet Dermatol 2008;7:189–193.
[19] Zhu W, Gao J. The use of botanical extracts as topical skin-lightening agents for the improvement of skin pigmentation disorders. J Investig Dermatol Symp Proc 2008;13:20–24.
[20] Kohen R. Skin antioxidants: their role in aging and in oxidative stress—new approaches for their evaluation. Biomed Pharmacother 1999;53:181–192.
[21] Takebayashi J, Tai A, Yamamoto I. pH-dependent long-term radical scavenging activity of AA-2G and 6-Octa-AA-2G against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation. Biol Pharm Bull 2003;26:1368–1370.
[22] Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005;53: 4290–4302.
[23] Waller JM, Maibach HI. Age and skin structure and function, a quantitative approach (I): blood flow, pH, thickness, and ultrasound echogenicity. Skin Res Technol 2005;11:221–235.
[24] Leong LP, Shui G. An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem 2002;76:69–75.
[25] Gillespie KM, Chae JM, Ainsworth EA. Rapid measurement of total antioxidant capacity in plants. Nat Protoc 2007;2: 867–870.
[26] Niki E, Komuro E, Takahashi M, Urano S, Ito E, Terao K. Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J Biol Chem 1988;263:19809–19814.
[27] Niki E, Noguchi N. Evaluation of antioxidant capacity. What capacity is being measured by which method? IUBMB Life 2000;50:323–329.
[28] Curto EV, Kwong C, Hermersdörfer H, Glatt H, Santis C, Virador V, Hearing VJ, Jr, Dooley TP. Inhibitors of mammalian melanocyte tyrosinase: *in vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochem Pharmacol 1999;57:663–672.
[29] Lynde CB, Kraft JN, Lynde CW. Topical treatments for melasma and postinflammatory hyperpigmentation. Skin Therapy Lett 2006;11:1–6.
[30] Draelos ZD. Skin lightening preparations and the hydroquinone controversy. Dermatol Ther 2007;20:308–313.

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