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Isolation and Tyrosinase Inhibitory Effects of Polyphenols from the Leaves of Persimmon, *Diospyros kaki*

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ABSTRACT: The main polyphenols were isolated from the leaves of six selected persimmon cultivars. Seven compounds were obtained by reverse-phase HPLC, and their structures were elucidated by multiple NMR measurements. These compounds are hyperoside, isoquercitrin, trifolin, astragalin, chrysontemin, quercetin-3-*O*-(2″-*O*-galloyl-*β*-D-glucopyranoside) (QOG), and kaempferol-3-*O*-(2″-*O*-galloyl-*β*-D-glucopyranoside) (KOG). Their inhibitory activity was tested against tyrosinase for the oxidation of L-DOPA, and only chrysontemin showed inhibitory activity. To investigate the differences of their inhibitory effects, the tyrosinase inhibitory activities of their aglycons, cyanidin, quercetin, and kaempferol, were also tested. As a result, it was confirmed that the most influential moiety for tyrosinase inhibition was the 3′,4′-dihydroxy groups of the catechol moiety. Moreover, the tyrosinase inhibitory activity of chrysontemin, which was identified in persimmon leaves for the first time, is supported by a simulated model of chrysontemin docking into mushroom tyrosinase.

KEYWORDS: *Diospyros kaki*, persimmon leaf extracts, polyphenols, tyrosinase, inhibitory mechanism, chrysontemin

INTRODUCTION

Persimmon (*Diospyros kaki*) originated in China and has been cultivated over hundreds of years throughout eastern Asia. In Japan, persimmon leaves are an abundant natural resource and have been utilized for wrapping foods to maintain their quality. They are also used as ingredients in health-promoting tea and traditional herbal medicines in China, Korea, and Japan.[1,2] In recent years, it has also been reported that persimmon leaf extracts are effective in microbial inhibition, radical scavenging, neuroprotection, blood pressure lowering, and thrombosis inhibition.[1,3−5] Persimmon leaves have attracted much attention due to their high polyphenol contents.[3,6] Polyphenols are being widely studied due to accumulating evidence of their beneficial properties on human health, including their antibacterial, anti-inflammatory, antiallergic, antimutagenic, and skin-lightening effects,[7,8] and hence for their potential as additives in the cosmetic and food industries.[8,9] The potential health risk of synthetic cosmetic and food additives has aroused great public concern. Therefore, there is greater demand than ever for safe and effective ingredients from natural resources.

Tyrosinase (EC 1.14.18.1) is a targeted enzyme for both the food and cosmetic industries.[10] Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones using the catalytic center formed by dinuclear copper (type 3 copper center).[11] In the food industry, tyrosinase is involved in the oxidation of phenolic compounds into highly reactive quinines, leading to the darkening of the products during processing or storage.[12] Although such enzymatic browning is favorable for certain foods, it usually leads to significant changes in appearance, flavor, and nutritional quality.[13] On the other hand, tyrosinase is also the rate-limiting enzyme for the formation of melanin, which is crucial for protecting the skin from ultraviolet radiation in animals. However, overproduction and accumulation of melanin in the skin can result in abnormal pigmentation disorders.[14] Therefore, compounds with tyrosinase inhibitory activity have the potential to be applied in both the food and cosmetic industries. Moreover, there have been a few reports that some polyphenols showed remarkable tyrosinase inhibitory activities.[15,16]

In the present work we isolated and characterized the main polyphenols from persimmon leaves using high-pressure liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and mass spectrometry (MS). We also analyzed the total phenolic content of the leaf extracts from six persimmon cultivars and characterized their major polyphenolic composition. In addition, we examined the tyrosinase inhibitory activities of the main polyphenols from persimmon leaves and investigated their inhibitory mechanism.

MATERIALS AND METHODS

Materials and Chemicals. The persimmon leaves from six kinds of cultivars [two general sweet cultivars, Fuyu (FY) and Jiro (JR); two sweet cultivars developed for enhanced color intensity in autumn (as a garnish for Japanese cuisine), Kinsyu (KS) and Tanrei (TR); and two general astringent cultivars, Yotsumizo (YM) and Saijo (SJ)] were supplied by the Japan National Institute of Fruit Tree Science at the beginning of October (green leaves) and the middle of November

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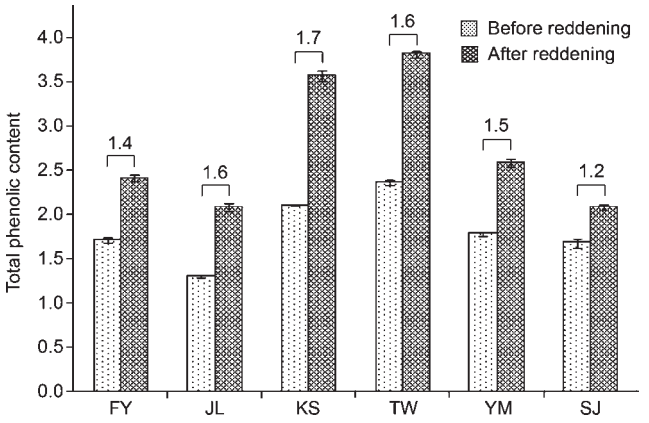


Figure 1. Total phenolic content of the leaf extracts before and after reddening. The increment in total phenolic content accompanying leaf reddening was expressed as a fold increase for each cultivar: Fuyu (FY), Jiro (JR), Kinsyu (KS), Tanrei (TR), Yotsumizo (YM), and Saijo (SJ).

(red leaves). The leaves were washed with tap water, dried in a dark place overnight, frozen, and stored at −20 °C for further use. All chemicals and solvents were purchased from Wako (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO).

Sample Preparation. One volume of persimmon leaves was added to 10 volumes of 80% methanol at room temperature. After homogenization, the mixture was stirred for 1 h at room temperature. After centrifugation for 10 min at 5000 rpm, the residue was re-extracted according to the same procedure. The coupled methanolic phases were mixed, filtered, and concentrated with a vacuum evaporator. After lyophilization, the powder was stored at −80 °C.

Total Phenolic Content Assay. The total phenolic content of persimmon leaves was measured using the Folin−Ciocalteu method[17] with some modifications. Briefly, 0.125 mL of a properly diluted sample (0.5−1.0 mg/mL in 50% ethanol) was added to 0.5 mL of distilled water and was mixed with 0.125 mL of the Folin−Ciocalteu phenol reagent. After 6 min of incubation at room temperature, 1.25 mL of 7% Na_2CO_3 and 3 mL of distilled water were added to the solution. The reaction mixtures were incubated for 90 min at room temperature, and then their absorbances at 760 nm were measured using a Shimadzu UV-2200A spectrophotometer (Shimadzu Corp., Kyoto, Japan). A calibration curve was prepared using a standard solution of gallic acid (0.1−0.5 mg/L, r^2 = 0.9994). The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight. All determinations were performed in triplicate (n = 3).

Analytical and Preparative HPLC. Analytical HPLC was used to identify the polyphenol compounds from each persimmon cultivar. Preparative HPLC was used for the separation and purification of polyphenol compounds from different persimmon leaves. HPLC analyses were carried out with a Shimadzu LC-VP HPLC system. Analyses of polyphenol compounds were performed on a DOCOSIL-B 4.6 ∅ × 250 mm Senshu Pack HPLC column (Senshu Scientific, Tokyo, Japan), whereas separations of polyphenol compounds were performed on a DOCOSIL-B 20 ∅ × 250 mm Senshu Pack HPLC column, equipped with a DOCOSIL-B 10 ∅ × 30 mm Senshu Pack HPLC guard column (Senshu Scientific). The flow rate and the injection volume of analytical HPLC were 1 mL/min and 20 μL of a 1 mg/mL sample, respectively. For preparative HPLC, the flow rate and the injection volume were 7 mL/min and 4 mL of a 5 mg/mL sample, respectively. The gradient of HPLC was from 10 to 80% methanol containing 0.3% trifluoroacetic acid (TFA), and the detection was carried out at 350 and 550 nm. Different polyphenol fractions were collected, concentrated, lyophilized, and stored at −80 °C for further use.

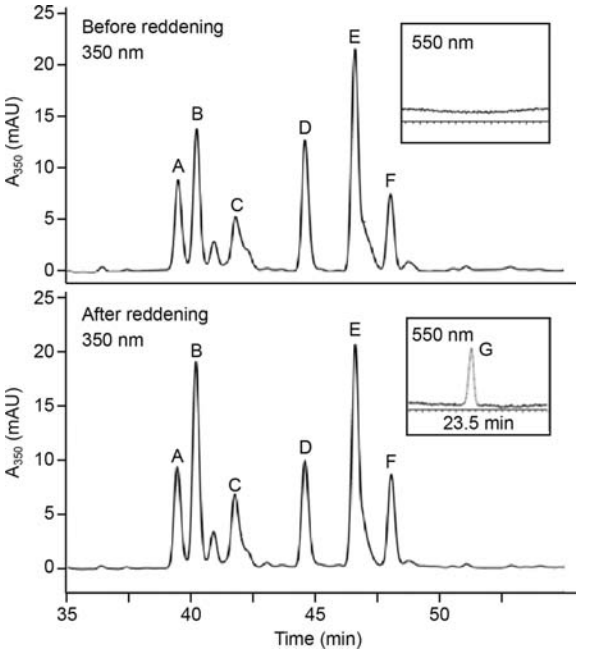


Figure 2. HPLC chromatograms of the leaf extracts from the persimmon cultivar Fuji (FY) at 350 and 550 nm. Components were identified as follows: A, quercetin-3-*O*-β-D-galactopyranoside (hyperoside); B, quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin); C, quercetin-3-*O*-(2″-*O*-galloyl-β-D-glucopyranoside) (QOG); D, kaempferol-3-*O*-β-D-galactopyranoside (trifolin); E, kaempferol-3-*O*-β-D-glucopyranoside (astragalin); F, kaempferol-3-*O*-(2″-*O*-galloyl-β-D-glucopyranoside) (KOG); G, cyanidin-3-*O*-β-D-glucopyranoside (chrysontemin). The data were collected by the interday.

NMR Measurements. Samples were loaded in 5 mm diameter NMR sample tubes with CD_3OD for quercetin-3-*O*-(2″-*O*-galloyl-β-D-glucopyranoside) (QOG) and kaempferol-3-*O*-(2″-*O*-galloyl-β-D-glucopyranoside) (KOG), and with D_2O for cyanidin-3-*O*-β-D-glucopyranoside (chrysontemin). NMR spectra were recorded on a JEOL JNM-α 500 NMR spectrometer (JEOL Ltd., Tokyo, Japan), a Varian Unity INOVA 500 NMR spectrometer (Varian Inc., Palo Alto, CA), and a Varian Unity INOVA 600 NMR spectrometer (Varian Inc.) at 25 °C. ^1H, ^{13}C one-dimensional (1D) NMR spectra and $^1H-^1H$ DQF-COSY, $^1H-^{13}C$ HSQC, and $^1H-^{13}C$ HMBC two-dimensional (2D) NMR spectra were used to identify constituents.

MS Measurements. All MS spectra were acquired in the positive ion mode using a Shimadzu AXIMA-CFR plus MALDI-TOF MS instrument. A 0.5 μL sample solution was applied onto the sample plate, followed by the addition of 0.5 μL of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in a mixture of acetonitrile and 0.1% v/v TFA, 50:50 v/v), and the resultant mixture was allowed to dry at room temperature.

Tyrosinase Inhibitory Assay. The tyrosinase inhibitory activity was measured as reported previously[18] with slight modifications. Briefly, the incubation mixture (total volume = 1 mL) consisted of 11 U of mushroom tyrosinase and 0.55 mM L-β-3,4-dihydroxyphenylalanine (L-DOPA), with or without isolated polyphenols of different concentrations in 50 mM phosphate buffer (pH 6.5). The mixture was incubated at 35 °C for 10 min, and then the absorbance was measured at 475 nm with a Shimadzu UV-2200A spectrophotometer. The test compounds were initially dissolved in DMSO and subsequently diluted to the appropriate concentrations with phosphate buffer. The final concentrations of DMSO in the final reaction mixture never exceeded 0.1% (v/v). The inhibitory activity of the sample is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC_{50}). The inhibition

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Components QOG, KOG, and Chrysontemin

QOG			KOG			chrysontemin		
chemical shift (ppm)			chemical shift (ppm)			chemical shift (ppm)		
^{1}H	^{13}C	assignment	^{1}H	^{13}C	assignment	^{1}H	^{13}C	assignment
3.78	62.5	C-6″	3.57, 3.77	62.5	C-6″	3.76	62.4	C-6″
3.42	71.6	C-4″	3.40	71.6	C-4″	3.28	71.1	C-4″
5.12	76.0	C-2″	5.09	75.9	C-2″	3.69	74.8	C-2″
3.64	76.4	C-3″	3.64	76.3	C-3″	3.81	78.1	C-3″
3.31	78.8	C-5″	3.32	78.7	C-5″	3.26	78.7	C-5″
6.32	94.5	C-8	6.34	94.5	C-8	6.92	95.0	C-6
6.15	99.7	C-6	6.16	99.6	C-6	5.28, 6.67	103.7	C-8,1″
5.25	100.5	C-1″	5.74	100.4	C-1″		113.3	C-10
	105.9	C-10		105.8	C-10	7.02	117.4	C-5′
7.11	110.5	C-3‴,7‴	7.11, 7.20	110.5	C-3‴,7‴	8.08	118.4	C-2′
6.82	116.1	C-2′	6.86	116.1	C-3′, 5′		121.2	C-1′
7.55	117.0	C-5′		121.5	C-2‴	8.26	128.3	C-6′
	121.5	C-2‴		122.8	C-1′	9.02	136.9	C-4
7.52	123.2	C-1′	7.95	132.0	C-2′,6′		145.6	C-3
7.52	123.4	C-6′		134.7	C-3		147.4	C-3′
	134.9	C-3		139.8	C-5‴		155.7	C-4′
	139.8	C-5‴		146.3	C-4‴,6‴		157.6	C-5
	146.0	C-4′		158.3	C-2,9		159.2	C-9
	146.3	C-4‴,6‴		161.4	C-4′		164.3	C-2
	149.7	C-3′		163.1	C-5‴		170.4	C-7
	158.2	C-2,9		165.6	C-7			
	163.2	C-5		167.6	C-1‴			
	165.7	C-7		179.1	C-4			
	167.8	C-1‴						
	179.1	C-4						

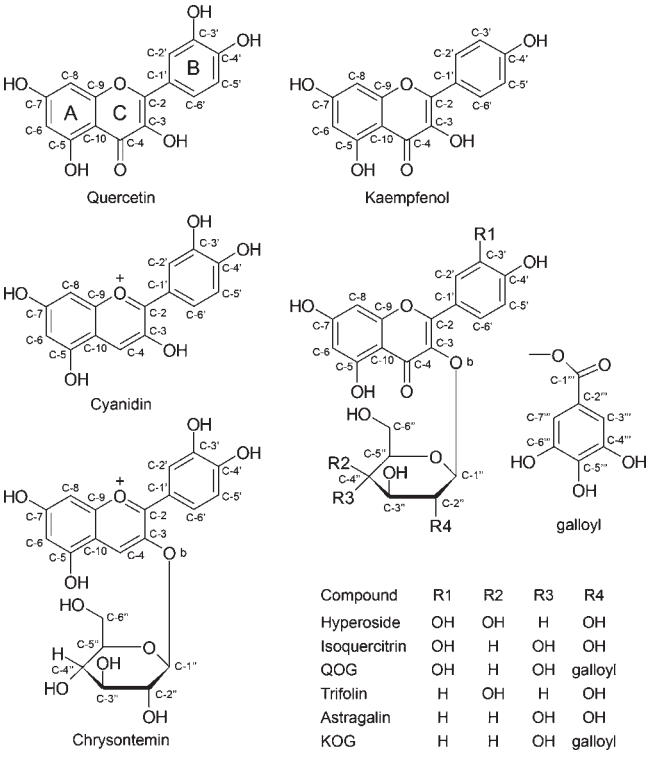
type was assayed by the Lineweaver−Burk plot, and the inhibition constant (K_i) was determined by the slope versus the concentration of the inhibitor.[19] All determinations were performed in triplicate ($n = 3$).

RESULTS

Total Phenolic Content of Persimmon Leaf Extracts. The total phenolic contents of each leaf extract (before and after reddening) are shown in Figure 1. The total phenolic contents of all red leaves were 1.2−1.7-fold higher than those of the original green leaves, and the largest contents of polyphenol were found in KS and TR, two sweet cultivars developed to produce more colorful leaves in autumn. In addition, the total phenolic contents of KS and TR leaves were high even though they were green, which means they are good sources for polyphenols.

Purification and Identification of Polyphenols from Persimmon Leaves. The analytical HPLC chromatograms of FY before and after reddening at 350 and 550 nm are shown in Figure 2. On the chromatogram detected by absorption at 350 nm, the peaks of the main polyphenols (A−F in Figure 2) were observed between 30 and 50 min (retention time) before and after reddening. Although the amounts of polyphenols were different, the patterns of the peaks were identical in all cultivars (data not shown). At the same time, a single peak (G in Figure 2) was observed at 23.5 min (retention time) on the chromatogram detected by absorption at 550 nm after reddening of the leaves of all cultivars except SJ. No additional peaks, which are possible to be characterized, were detected by the absorptions at 280 and 254 nm. Compared with the retention time and the m/z of the standard compounds, quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside, peak A), quercetin-3-*O*-*β*-D-glucopyranoside (isoquercitrin, peak B), kaempferol-3-*O*-*β*-D-galactopyranoside (trifolin, peak D), and kaempferol-3-*O*-*β*-D-glucopyranoside (astragalin, peak E) were identified, which is consistent with previous studies.[1,20,21] To identify peaks C, F, and G, ^{1}H and ^{13}C 1D NMR spectra and $^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HMBC, and $^{1}H-^{1}H$ DQF-COSY 2D NMR spectra were measured, following the confirmation of the molecular weights by the MALDI-TOF MS spectra. These analyses showed that the C, F, and G peaks were quercetin-3-*O*-(2″-*O*-galloyl-*β*-D-glucopyranoside) (QOG), kaempferol-3-*O*-(2″-*O*-galloyl-*β*-D-glucopyranoside) (KOG), and cyanidin-3-*O*-*β*-D-glucopyranoside (chrysontemin), respectively. The chemical shift values for QOG, KOG, and chrysontemin are listed in Table 1, and the chemical structures of the identified polyphenols are shown in Figure 3. The peak between peaks B and C could not be characterized in this study because it was minor compared with the other peaks. Although the ^{1}H and ^{13}C chemical shift is a little different, which is probably caused by the different solvent used for NMR measurements, the identification of QOG and KOG in persimmon leaves is consistent with a previous paper,[22] and this is the first time that chrysontemin has been identified in persimmon leaves. However, chrysontemin has been reported to be the major anthocyanin in autumn leaves of various plant species.[23−25]

Quantitative Comparison of the Polyphenols from Six Cultivars. The amounts of the purified polyphenols in each persimmon cultivar, which were calculated from the corresponding HPLC spectra, were expressed as micromoles per milligram of leaf extracts and are shown in Table 2. The increments of the polyphenols after reddening in each persimmon cultivar are shown in Figure 4. As described above, no chrysontemin was detected in the autumn leaves of SJ (Table 2). Chrysontemin was the only red-colored compound in the main polyphenols extracted from persimmon leaves. In contrast, the color of the autumn leaves of SJ is yellow rather than red, which means that chrysontemin is the main colorant for the spectacular autumn red in most persimmon cultivars. The autumn yellow is caused by the loss of chlorophyll, which allows the yellow carotenoid pigments to be revealed.[26,27] In the present study, although the total amounts of polyphenols increased by various degrees, some of the polyphenols, such as the hyperoside in FY, JR, and YM, decreased markedly after reddening. Because the biosyntheses of anthocyanins and flavonoids share the same upstream pathway,[28,29] it is possible that the levels of the nutrients required for the biosynthesis of hyperoside are not sufficient in the autumn leaves of the cultivars FY, JR, and YM. At the same time, the biosynthesis of other flavonoids and anthocyanins that could reduce photoinhibition and photodamage in autumn leaves[28] is predominant. On the other hand, for cultivars KS and TR, which are sweet cultivars developed for enhanced color intensity in autumn, all of the polyphenols were found to be increased after reddening.



Compound	R1	R2	R3	R4
Hyperoside	OH	OH	H	OH
Isoquercitrin	OH	H	OH	OH
QOG	OH	H	OH	galloyl
Trifolin	H	OH	H	OH
Astragalin	H	H	OH	OH
KOG	H	H	OH	galloyl

Figure 3. Chemical structures of the polyphenols from persimmon leaf extracts and their aglycons.

Tyrosinase Inhibitory Activity of the Polyphenols. We examined the tyrosinase inhibitory activities of the main polyphenols obtained from the persimmon leaves for the oxidation of L-DOPA. Chrysontemin showed tyrosinase inhibitory activity

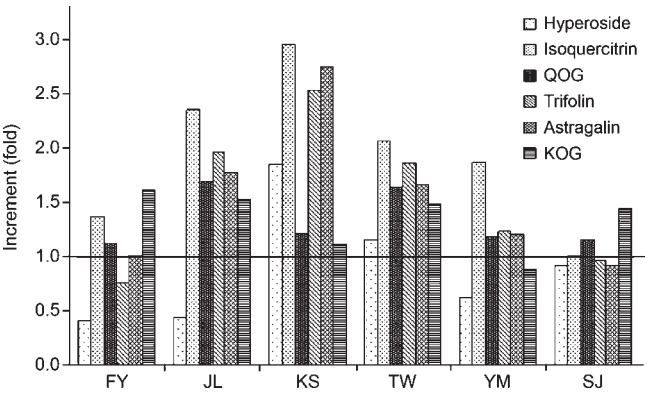


Figure 4. Comparison of the amounts of the isolated polyphenols from six persimmon cultivars, Fuyu (FY), Jiro (JR), Kinsyu (KS), Tanrei (TR), Yotsumizo (YM), and Saijo (SJ), before and after reddening. The polyphenol amounts before reddening were normalized as 1.0.

Table 2. Amounts of the Purified Polyphenols from Each Persimmon Cultivar, Fuyu (FY), Jiro (JR), Kinsyu (KS), Tanrei (TR), Yotsumizo (YM), and Saijo (SJ), before and after Reddening[a]

	hyperoside	isoquercitrin	QOG	trifolin	astragalin	KOG	chrysontemin
				Before Reddening			
FY	12.1 ± 0.2	21.3 ± 0.2	12.1 ± 0.2	14.5 ± 0.1	25.0 ± 0.2	10.7 ± 0.1	NF[b]
JR	11.9 ± 0.2	28.1 ± 0.6	17.0 ± 0.4	10.8 ± 0.1	26.6 ± 3.6	14.3 ± 0.1	NF
KS	5.7 ± 0.1	12.3 ± 0.2	12.9 ± 0.5	8.0 ± 0.3	16.0 ± 0.1	16.9 ± 0.4	NF
TR	8.0 ± 0.1	21.5 ± 0.1	10.8 ± 0.6	9.5 ± 0.2	26.2 ± 3.5	14.8 ± 0.1	NF
YM	10.9 ± 0.5	22.2 ± 1.0	13.1 ± 0.9	12.7 ± 0.5	31.8 ± 0.9	20.2 ± 0.3	NF
SJ	10.1 ± 0.3	27.2 ± 0.4	17.4 ± 0.4	13.0 ± 0.5	38.5 ± 0.7	14.5 ± 0.3	NF
				After Reddening			
FY	4.9 ± 1.0	29.5 ± 0.2	13.6 ± 0.1	11.1 ± 0.2	25.4 ± 2.5	17.4 ± 3.1	4.9 ± 1.0
JR	5.3 ± 0.9	66.8 ± 0.8	29.1 ± 0.7	21.4 ± 0.4	47.6 ± 5.1	22.0 ± 0.3	5.3 ± 0.9
KS	10.6 ± 0.4	36.8 ± 0.5	15.9 ± 0.9	20.4 ± 0.5	44.5 ± 0.1	19.0 ± 0.2	10.6 ± 0.4
TR	9.3 ± 2.7	44.9 ± 0.7	17.9 ± 1.2	17.9 ± 0.2	44.1 ± 5.7	22.3 ± 0.7	9.3 ± 2.7
YM	6.9 ± 0.3	41.9 ± 1.0	15.8 ± 0.6	15.7 ± 0.2	38.8 ± 3.9	18.1 ± 0.4	6.9 ± 0.3
SJ	9.3 ± 0.1	27.7 ± 0.2	20.3 ± 0.2	12.6 ± 0.1	35.6 ± 3.8	21.0 ± 0.2	NF

[a] The amounts of the purified polyphenols are expressed as μmol/mg leaf extracts. [b] Not found.

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Table 3. Tyrosinase Inhibitory Activity of Isolated Compounds and Contrast Compounds: Cyanidin, Quercetin, and Kaempferol

compound	IC_{50} (μM)	inhibition type	inhibition constant K_i (μM)
cyanidin	9.1 ± 0.1	competitive	5.2 ± 0.2
quercetin	9.7 ± 0.3	competitive	3.8 ± 0.3
kaempferol	50.1 ± 0.5	competitive	30.2 ± 6.8
chrysontemin	211 ± 2	competitive	192 ± 3
hyperoside	ND[a]	NT[b]	NT
isoquercitrin	ND	NT	NT
QOG	ND	NT	NT
trifolin	ND	NT	NT
astragalin	ND	NT	NT
KOG	ND	NT	NT

[a] Not detected. [b] Not tested.

(IC_{50} = 211 ± 2 μM), and the other polyphenols, hyperoside, isoquercitrin, QOG, trifolin, astragalin, and KOG, did not exhibit any inhibitory activity against the L-DOPA oxidation up to 1 mg/mL (>1.6 mM). Chrysontemin, isoquercitrin, and astragalin share a similar structure, which includes a common glucopyranoside moiety at the C-3 position of the flavonoid moiety. To study the inhibition mechanism of these polyphenols, the tyrosinase inhibitory activities of their aglycons, that is, cyanidin, quercetin, and kaempferol, were also tested. The tyrosinase inhibitory activity (IC_{50}) and inhibition constant (K_i) are shown in Table 3. Quercetin is a well-known positive control.[11,32] The tyrosinase activity was effectively reduced by cyanidin (IC_{50} = 9.07 ± 0.10 μM) and quercetin (IC_{50} = 9.67 ± 0.30 μM) and, to a lesser extent, also by kaempferol (IC_{50} = 50.1 ± 0.3 μM). The inhibition constants (K_i) for cyanidin, quercetin, kaempferol, and chrysontemin were 5.15 ± 0.20, 3.80 ± 0.30, 30.2 ± 6.8, and 192 ± 3 μM, respectively. The tyrosinase inhibitions of these polyphenols belong to the competitive type, and the Lineweaver−Burk plots are shown in Figure 5. Cyanidin and quercetin inhibited tyrosinase to a similar degree and in a similar manner, which means that the inhibitory effect of chrysontemin could not be explained only by the inhibitory activity of the flavonoid moiety.

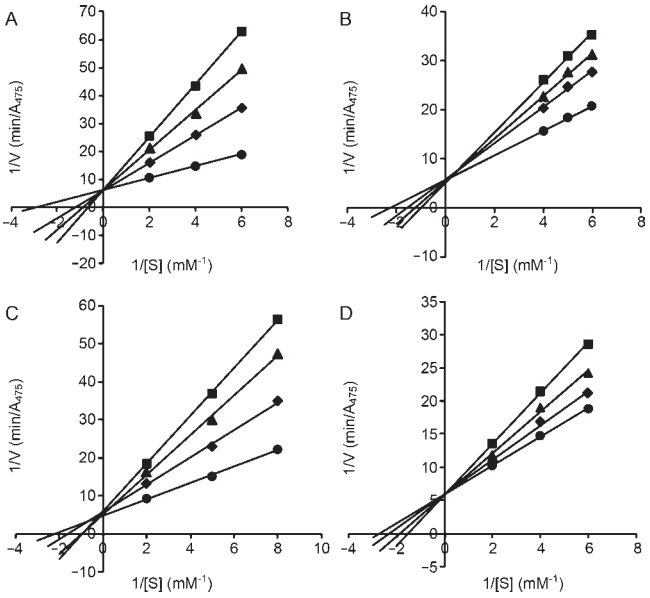

Figure 5. Lineweaver−Burk plots for the catalysis of L-DOPA by mushroom tyrosinase: concentrations of each tested polyphenol for plots (A) quercetin, 0, 4, 8, and 12 μM; (B) kaempferol, 0, 10, 20, and 30 μM; (C) cyanidin, 0, 4, 8, and 12 μM; (D) chrysontemin, 0, 50, 100, and 150 μM.

DISCUSSION

Our results showed that cyanidin, quercetin, kaempferol, and chrysontemin are all competitive inhibitors (Figure 5), which is consistent with a previous paper[30] in which the most common inhibitory mode of flavonoid inhibitors was shown to be competitive inhibition against the oxidation of L-DOPA by tyrosinase. Tyrosinase has a catalytic center formed by binuclear copper (type 3 copper center).[11] The inhibitory mechanism of polyphenols was proposed to be associated with their chelation abilities toward binuclear copper.[31−33] Cyanidin and quercetin showed strong inhibitory effects, whereas kaempferol showed a little bit lower inhibitory activity (Table 3). In quercetin, there are three commonly cited metal-binding sites: the catechol moiety with 3′- and 4′-hydroxy groups on the B ring, the 4-keto and 3-hydroxy groups of the C ring, and the 4-keto and 5-hydroxy groups of the C and A rings.[34,35] The decreased inhibitory activity of kaempferol relative to quercetin is caused by the loss of a 3′-hydroxy group (Figure 3). In addition, cyanidin exerted inhibitory activity almost equal to that of quercetin without the 4-keto group that constitutes two putative chelation sites of the A and C rings. These findings indicate that the catechol moiety with 3′,4′-dihydroxy groups on the B ring is the most important region for copper chelation.

The flavonoid glycosides obtained from persimmon leaves hardly showed any tyrosinase inhibitory activity, which is consistent with two previous papers.[32,36] One possible reason for the decreased inhibitory activity would be the loss of the 3-hydroxyl group by substitution of the glycoside moiety (Figure 3). However, we can rule this explanation out, because we considered that the 3′,4′-dihydroxy groups on the B ring are the most important region for copper chelation. The other reason is the steric hindrance effect of the bulky glycoside moiety, which prevents the inhibitor from fitting into the active site of the enzyme.[30] Only chrysontemin showed tyrosinase inhibitory activity (IC_{50} = 211 ± 2 μM) in the flavonoid glycosides tested in this study and showed a competitive inhibitory mode against tyrosinase (Figure 5). The only difference between the structure of isoquercitrin and that of chrysontemin is the 4-keto group of the C ring, which seems to be responsible for the distinct inhibitory activities against tyrosinase. It seems that the 3′,4′-dihydroxy groups of isoquercitrin, with the 4-keto group on the C ring, cannot be fit into the active site of the enzyme due to the steric hindrance effects. Likewise, the steric hindrance effects in hyperoside and QOG would prevent their 3′,4′-dihydroxy groups from fitting into the active site of the enzyme. In contrast, the 3′,4′-dihydroxy groups of chrysontemin can fit into the active site, although not as readily as the aglycon of chrysontemin, cyanidin.

To understand the mechanism underlying the inhibition of tyrosinase by chrysontemin, the docking modes of chrysontemin are examined in the catalytic sites of tyrosinase. Although several crystal structures of tyrosinase are available from PDB, the crystal structure of octopus hemocyanin (PDB code 1js8), which shares 39.1% sequence identity with mushroom tyrosinase and also

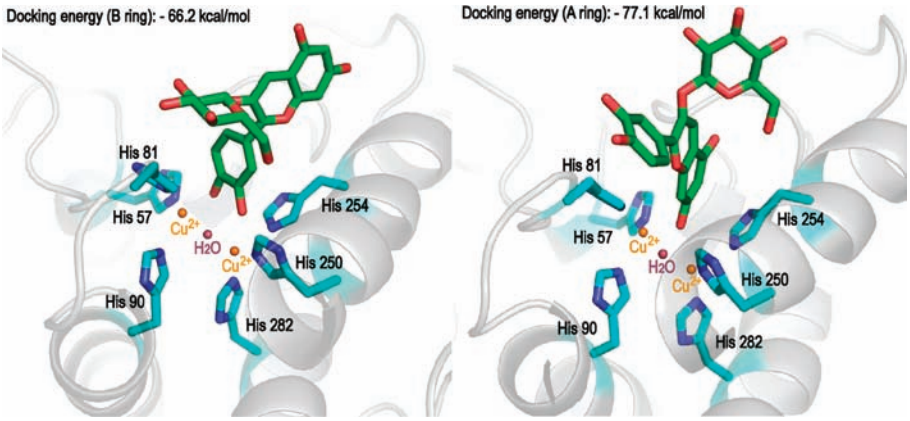

Figure 6. Docking model for chrysontemin with mushroom tyrosinase. The homology model of mushroom tyrosinase was conducted using SWISS-MODEL, and the crystal structure of octopus hemocyanin (PDB code, 1js8; sequence identity, 39.1%) was used as the template molecule. The dihydroxy phenyl (B ring) of chrysontemin (green) chelates the catalytic dicopper center (cyan) of tyrosinase (left). The A ring of chrysontemin can also enter into the active site of tyrosinase (right).

belongs to the type 3 copper proteins, was used as the template molecule to obtain the structure of the mushroom tyrosinase. The structure model that was created using a SWISS-MODEL automated protein-modeling software[37] provided the best docking model with quercetin, which well explained the chelation abilities of 3′- and 4′-hydroxy groups toward binuclear copper of the enzyme (data not shown). The docking simulation was performed on ASEDock of the Molecular Operation Environment (MOE) with the structure model of the mushroom tyrosinase and chrysontemin, using the MMFF94x force field for energy minimization. As shown in Figure 6, inhibition by chrysontemin of the mushroom tyrosinase is caused by the docking of 3′,4′-dihydroxyphenyl (B ring) chelating to the coppers in the active site (docking energy = −66.2 kcal/mol), and each copper atom is coordinated by three histidines, which are highly conserved throughout all type 3 copper proteins. However, it is easier for the A ring of chrysontemin to dock into the active site of tyrosinase (docking energy = −77.1 kcal/mol). The affinity of 3′,4′-dihydroxyphenyl (B ring) chelator to the active site is considered to be weakened by the steric hindrance effect of the glycoside moiety because the inhibitory effects of flavonoid glycosides are quite weak compared with that of aglycons. However, chrysontemin would be able to exert the inhibitory activity toward the mushroom tyrosinase by the A or B ring docking into the active site, which is permitted due to the absence of a 4-keto group.

Kojic acid is a traditional depigmenting agent and shows excellent tyrosinase inhibitory activity. However, this agent carries important safety risks, including cytotoxicity, skin cancer, and dermatitis, under long-term exposure.[38] Therefore, there is an ongoing demand for safe and effective tyrosinase inhibitors of natural origin. In the present study, seven polyphenols were successfully isolated and purified from the leaves of six persimmon cultivars. Among the seven obtained polyphenols, only chrysontemin showed moderate inhibitory activity for tyrosinase, and there are reports that chrysontemin inhibits tumor promoter-induced carcinogenesis and tumor metastasis in vivo.[39,40] In addition, the other obtained compounds are already well documented to exhibit different activities, such as the antifungal activity of hyperoside and trifolin;[41] the anti-inflammatory activity of isoquercitrin;[42] the antiallergic activity of astragalin;[43] and the angiotensin-converting enzyme inhibitory activities of astragalin, isoquercitrin, QOG, and KOG.[22] Therefore, it is clear that the polyphenols from persimmon leaves are worthy of further study as potential pharmaceutical ingredients.

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REFERENCES

(1) Yamada, Y.; Yamamoto, A.; Yoneya, N.; Nakatani, N. Identification of kaempferol from the leaves of *Diospyros kaki* and its antimicrobial activity against *Streptococcus mutans*. *Biocontrol Sci.* **1999**, *4*, 97–100.

(2) Chen, G.; Wei, S. H.; Huang, J.; Sun, J. A novel C-glycosylflavone from the leaves of *Diospyros kaki*. *J. Asian Nat. Prod. Res.* **2009**, *11*, 503–507.

(3) Bei, W.; Zang, L.; Guo, J.; Peng, W.; Xu, A.; Good, D. A.; Hu, Y.; Wu, W.; Hu, D.; Zhu, X.; Wei, M.; Li, C. Neuroprotective effects of a standardized flavonoid extract from *Diospyros kaki* leaves. *J. Ethnopharmacol.* **2009**, *126*, 134–142.

(4) Sa, Y. S.; Kim, S. J.; Choi, H. S. The anticoagulant fraction from the leaves of *Diospyros kaki* L. has an antithrombotic activity. *Arch. Pharm. Res.* **2005**, *28*, 667–674.

(5) Sakanaka, S.; Tachibana, Y.; Okada, Y. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food Chem.* **2005**, *89*, 4569–4575.

(6) Lee, N. K.; Son, K. H.; Chang, H. W.; Kang, S. S; Park, H.; Heo, M. Y.; Kim, H. P. Prenylated flavonoids as tyrosinase inhibitors. *Arch. Pharm. Res.* **2004**, *27*, 1132–1135.

(7) Alan, L.; Miller, M. D. Antioxidant flavonoids: structure, function and clinical usage. *Alt. Med. Rev.* **1996**, *1*, 103–111.

(8) Kiefer, S.; Weibel, M; Smits, J.; Juch, M.; Tiedtke, J.; Herbst, N. Citrus flavonoids with skin lightening effects − safety and efficacy studies. *SOFW* **2010**, *136*, 45–54.

(9) Hanamura, T.; Uchida, E.; Aoki, H. Skin-lightening effect of a polyphenol extract from acerola (*Malpighia emarginata* DC.) fruit on UV-induced pigmentation. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 3211–3218.

(10) Mallavadhani, U. V.; Panda, A. K.; Rao, Y. R. Pharmacology and chemotaxonomy of *Diospyros*. *Phytochemistry* **1998**, *49*, 901–951.

(11) Chang, T. An updated review of tyrosinase inhibitors. *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475.

(12) Chiari, M. E.; Joray, M. B.; Ruiz, G.; Palacios, S. M.; Carpinella, M. C. Tyrosinase inhibitory activity of native plants from central

Argentina: isolation of an active principle from *Lithrea molleoides*. *Food Chem.* **2010**, *120*, 10–14.

(13) Gawlik-Dziki, U.; Zlotek, U.; Swieca, M. Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. *capitata* L.). *Food Chem.* **2008**, *107*, 129–135.

(14) Qiu, L.; Chen, Q. H.; Zhuang, J. X.; Zhong, X.; Zhou, J. J.; Guo, Y. J. Inhibitory effects of α-cyano-4-hydroxycinnamic acid on the activity of mushroom tyrosinase. *Food Chem.* **2009**, *112*, 609–613.

(15) Jeong, S. H.; Ryu, Y. B.; Curtis-Long, M. J.; Ryu, H. W.; Baek, Y. S.; Kang, J. E.; Lee, W. S.; Park, K. H. Tyrosinase inhibitory polyphenols from roots of *Morus lhou*. *J. Agric. Food Chem.* **2009**, *57*, 1195–1203.

(16) Sirat, H. M.; Rezali, M. F.; Ujang, Z. Isolation and identification of radical scavenging and tyrosinase inhibition of polyphenols from *Tibouchina semidecandra* L. *J. Agric. Food Chem.* **2010**, *58*, 10404–10409.

(17) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144–158.

(18) Vanni, A.; Gastaldi, D.; Giunata, G. Kinetic investigations on the double enzymatic activity of the tyrosinase mushroom. *Ann. Chim.* **1990**, *80*, 35–60.

(19) Wang, Q.; Qiu, L.; Chen, X. R.; Song, K. K.; Shi, Y.; Chen, Q. X. Inhibitory effects of phloridzin dihydrate on the activity of mushroom (*Agaricus bisporus*) tyrosinase. *Bioorg. Med. Chem.* **2007**, *15*, 1568–1571.

(20) Tsurunaga, Y.; Matsumoto, T.; Kurahashi, T.; Mochida, K.; Suzuki, Y.; Itamura, H. Functional component contents in mature leaves, young shoots, and adventitious shoots of Japanese persimmon 'Saijo'. *Food Sci. Technol. Res.* **2008**, *14*, 62–66.

(21) Chen, G.; Lu, H.; Wang, C.; Yamashita, K.; Manabe, M.; Meng, Z.; Xu, S.; Kodama, H. Effect of five flavonoid compounds isolated from leaves of *Diospyros kaki* on stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. *Clin. Chim. Acta* **2002**, *326*, 169–175.

(22) Kameda, K.; Takaku, T.; Okuda, H.; Kimura, Y.; Okuda, T.; Hatano, T.; Agata, I.; Arichi, S. Inhibitory effects of various flavonoids isolated from leaves of persimmon on angiotensin-converting enzyme activity. *J. Nat. Prod.* **1987**, *50*, 680–683.

(23) Ishikura, N. Anthocyanins and other phenolics in autumn leaves. *Phytochemistry* **1972**, 2555–2558.

(24) Iwashina, T. Detection and distribution of chrysanthemin and idaein in autumn leaves of plants by high performance liquid chromatography. *Ann. Tsukba Bot. Gard.* **1996**, *15*, 1–18.

(25) Ji, S. B.; Yokoi, M.; Saito, N.; Mao, L. S. Distribution of anthocyanins in Aceraceae leaves. *Biochem. Syst. Ecol.* **1992**, *20*, 771–781.

(26) Lee, D. W. Anthocyanins in autumn leaf senescence. *Adv. Bot. Res.* **2002**, *37*, 147–165.

(27) Field, T. S.; Lee, D. W.; Holbrook, N. M. Why leaves turn red in autumn, the role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiol.* **2001**, *127*, 566–574.

(28) Winkel-Shirley, B. Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* **2001**, *126*, 485–493.

(29) He, F.; Mu, L.; Yan, G.; Liang, N.; Pan, Q.; Wang, J.; Reeves, M. J.; Duan, C. Biosynthesis of anthocyanins and their regulation in colored grapes. *Molecules* **2010**, *15*, 9057–9091.

(30) Mayer, A. M. Polyphenol oxidases in plants − recent progress. *Phytochemistry* **1986**, *26*, 11–20.

(31) Tsuda, T.; Osawa, T. Inhibition of tyrosinase activity by the anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Food Sci. Technol. Int. Tokyo* **1997**, *3*, 82–83.

(32) Kubo, I.; Kinst-Hori, I. Flavonols from saffron flowers: tyrosinase activity and inhibition mechanism. *J. Agric. Food Chem.* **1999**, *47*, 4121–4125.

(33) Kim, D.; Park, J.; Kim, J.; Han, C.; Yoon, J.; Kim, N.; Seo, J.; Lee, C. Flavonoids as mushroom tyrosinase inhibitors: a fluorescence quenching study. *J. Agric. Food Chem.* **2006**, *54*, 935–941.

(34) Engelmann, M. D.; Hutcheson, R.; Cheng, I. F. Stability of ferric complexes with 3-hydroxyflavones/(flavonol), 5,7-dihydroxyflavone (chrysin) and 3′,4′-dihydroxyflavone. *J. Agric. Food Chem.* **2005**, *53*, 2953–2960.

(35) Triantafyllou, A.; Mylonis, I.; Simos, G.; Bonanou, S.; Tsakalof, A. Flavonoids induce HIF-1α but impair its nuclear accumulation and activity. *Free Radical Biol. Med.* **2008**, *44*, 657–670.

(36) Karioti, A.; Protopappa, A.; Megoulas, N.; Skaltsa, H. Identification of tyrosinase inhibitors from *Marrubium velutinum* and *Marrubium cylleneum*. *Bioorg. Med. Chem.* **2007**, *15*, 2708–2714.

(37) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modeling. *Bioinformatics* **2006**, *22*, 195–201.

(38) Tiedtke, J.; Morel, J.; Marks, O. Depigmentation factor bioflavonoids − a safe and effective skin lightener based on encapsulated citrus bioflavonoids. *Cosmetochemistry* **2004**, 12–17.

(39) Ding, M.; Feng, R.; Wang, S. Y.; Bowman, L.; Lu, Y.; Qian, Y.; Castranova, V.; Jiang, B. H.; Shi, X. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* **2006**, *281*, 17359–17368.

(40) Xu, M.; Bower, K. A.; Wang, S.; Frank, J. A.; Chen, G.; Ding, M.; Wang, S.; Shi, X.; Ke, Z. J.; Luo, J. Cyanidin-3-glucoside inhibits ethanol-induced invasion of breast cancer cells overexpressing ErbB2. *Mol. Cancer* **2010**, *9*, 285–298.

(41) Li, S.; Zhang, Z.; Cain, A.; Wang, B.; Long, M.; Taylor, J. Antifungal activity of camptothecin, trifolin, and hyperoside isolated from *Camptotheca acuminata*. *J. Agric. Food Chem.* **2005**, *53*, 32–37.

(42) Rogerio, A. P.; Kanashiro, A.; Fontanari, C.; da Silva, E. V. G.; Lucisano-Valim, Y. M.; Soares, E.; Ge Faccioli, L. H. Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. *Inflamm. Res.* **2007**, *56*, 402–408.

(43) Kotani, M.; Matsumoto, M.; Fujita, A.; Higa, S.; Wang, W.; Suemura, M.; Kishimoto, T.; Tanaka, T. Persimmon leaf extract and astragalin inhibit development of dermatitis and IgE elevation in NC/Nga mice. *J. Allergy Clin. Immunol.* **2000**, *106*, 59–166.