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Simple Detection Method of Powerful Antiradical Compounds in the Raw Extract of Plants and Its Application for the Identification of Antiradical Plant Constituents

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A simple detection method for a powerful radical scavenging compound in a mixture containing a large variety of compounds, such as the raw extract of edible plants, was developed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as the radical reagent. The method was established on the basis of the features of the typical chain-breaking antioxidation reaction mechanism, which suggests that the radical scavenging antioxidant should be converted to other stable nonradical compounds during the reaction. This method requires only a simple HPLC instrument, and the disappearance or decrease in the peak intensity, which is induced by the addition of DPPH. This change is monitored by the HPLC to detect the powerful radical scavenger from the complex mixture. The method was applied to the detection and identification of the most powerful antiradical compound in the extracts of three antioxidatively active plant extracts (*Psidium guajava*, *Citrus depressa*, and *Hypericum chinense*). The radical scavenging efficiency of a newly identified compound from *H. chinense* was also compared with that of Trolox and catechin using the method.

KEYWORDS: Radical scavenger; antioxidant detection; DPPH; HPLC; *Psidium guajava*; *Citrus depressa*; *Hypericum chinense*

INTRODUCTION

An antioxidant, an inhibitor for the oxidation of biomolecules, is very important not only for food preservation but also for the defense of a living system against oxidative stress (*1*). The oxidation of a biomolecule usually occurs via an autoxidation mechanism, which includes the radical chain reaction of the peroxyl radical of a biomolecule (*2*). It is well-known that a potent antioxidant has a significant peroxyl radical scavenging ability by donation of its hydrogen atom to the radical species. Food scientists have sought new useful antioxidants in the big plant kingdom including vegetables, crops, spices, and also medicinal herbs (*3*−*6*). Plants accumulate efficient antioxidative compounds such as polyphenols in the body. Their isolation and the identification of their chemical structures are very important for this application as new food additives. Although many isolation attempts have been carried out so far, a successful isolation usually requires a repeated assay-guided fractionation process. This repeated process not only decomposes the antioxidant but is also very time-consuming. The development of an effective method to find strong antioxidants in the raw extract of plants should accelerate these isolation studies. Recently, an on-line HPLC method for the detection of an antioxidant using a purple radical species, 1,1-diphenyl-2-picrylhydrazyl (DPPH), has been developed (*7*, *8*). This method has many advantages over the classical assay-guided fractionation method. The separation and detection of an antioxidant are completed at the same time, and the DPPH is inexpensive and easy to handle (*9*). Also, special equipment for the reaction of the antioxidant with DPPH and one more detector for the measurement of the decrease of DPPH are required. In addition to these items, it is not easy to find the most active and most contributing antioxidant in the raw extract of the plant. Because plant extracts consist of a mixture of many kinds of phenolics, which have higher or lower antioxidant capacities, the conditioning for selective detection is slightly complicated.

Common antioxidant activity is a typical activity based on a chemical reaction. Its process is shown in the following reaction equations:

$$S{-}OO\cdot + AH \rightarrow S{-}OOH + A\cdot \quad (1)$$

$$A\cdot \rightarrow \text{nonradical compounds} \quad (2)$$

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In eqs 1 and 2, S is the oxidation substrate, AH is the antioxidant, and A· is the antioxidant radical.

The second equation suggests very useful insight, which shows that the antioxidant must convert to another compound during the antioxidation process (*10*, *11*). The plant extract has various constituents; however, only the antioxidatively active constituents (AH) should be converted to A·, when a radical species such as S−OO· was added to the extract. The produced A· was then converted to nonradical compounds with chemical structures different from that of the original AH. A recent HPLC technique can separate most of the plant constituents as individual peaks. Only peaks corresponding to the strong antioxidants (AH) would disappear or change to other peaks by the reaction with the added radical species. Therefore, the powerful antioxidative constituent would be detected as the corresponding decreased or lost peak on the HPLC chromatogram. This method can easily control the radical reaction by regulating the amount of the added radical species, reaction time, and reaction temperature, similar to normal organic reaction. Under the appropriate reaction conditions, a more active compound can be detected as a faster reducing peak according to its reactivity, which may enable one to select the most active compound in the raw extract by only using this simple HPLC instrument. We now report the detailed procedure of this method using DPPH as a radical species and some successful examples for the identification of the powerful anti-DPPH-radical compounds from three antioxidatively active plant extracts (*12*).

MATERIALS AND METHODS

Chemicals and Instruments. DPPH was purchased from Wako Pure Chemicals (Osaka, Japan). Ellagic acid was obtained from Aldrich Japan (Tokyo, Japan). (+)-Catechin was purchased from Nacalai Tesque (Kyoto, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Tokyo Kasei (Tokyo, Japan). All solvents and other reagents were obtained from Nacalai Tesque as extra pure grade. The NMR spectra were measured using a Unity Plus 500 spectrometer (500 MHz, Varian, Palo Alto, CA) or an EX-400 spectrometer (400 Hz, JEOL, Tokyo, Japan). Mass spectra were measured with an SX-102A spectrometer (JEOL). IR spectra were measured with an FTIR-8400 spectrometer (Shimadzu, Kyoto, Japan) by the dry film method. The analytical HPLC was performed with an LC-10 low-pressure gradient system (Shimadzu) consisting of an LC-10ATvp pump, a CTO-10Avp column oven, and a DAD (SPD-M10Avp). Preparative HPLC was performed with an LC-6AD recycle system (Shimadzu) equipped with a UV-8011 detector (Tosoh, Tokyo, Japan).

Plant Materials. The leaves of guava (*Psidium guajava*), the stems of hirami lemon (*Citrus depressa*), and the stems of byoyanagi (*Hypericum chinense*) were collected from Nakazen Farm (Chinen, Okinawa, Japan) in September 2001. All plant materials were dried at 60 °C for 12 h by a circulatory dryer (SP-A, Sansyu, Tokyo, Japan).

Extraction of Plant and Preparation of Analytical Solution. The dried plant material (30 g) was ground to a powder and then extracted with 99% ethanol (350 mL) for 6 days at room temperature. After filtration, the filtrate was evaporated in vacuo. Twenty-five milligrams of the residue was dissolved in methanol (2 mL) and passed through a Sep-Pak Plus C18 cartridge (Waters, Milford, MA). The cartridge was successively washed with 1 mL of methanol and 1 mL of acetonitrile. All eluates were combined to make the analytical solution (4 mL). The solution was stored at −30 °C until required.

Detection Procedure of Antiradical Compounds in Plant Extract by HPLC. To the analytical solution (100 μL) was added the appropriate amount (50−100 μL) of freshly prepared 5 mM DPPH methanol solution. The mixture was well stirred and allowed to stand for 3 min at 25 °C. The solution (15−20 μL) was injected into the HPLC and analyzed under the following conditions: column, ODS-80Ts (150 × 4.6 mm i.d.); solvent A, 0.1% H_3PO_4; solvent B, CH_3CN; gradient systems, a linear gradient from 10% of solvent B to 40% of solvent B (40 min) and then 100% of solvent B (60 min) for the extracts from *H. chinense* and *C. depressa* or a linear gradient from 10% of solvent B to 20% of solvent B (20 min) and then 100% of solvent B (70 min) for the extract from *P. guajava*; flow rate, 0.5 mL/min; detection, 254 nm. Reference HPLC data were obtained by direct injection of each analytical solution (10 μL).

Identification of Ellagic Acid (1) in the Extract of *P. guajava*. The identification was carried out by the co-injection method in the HPLC analysis. Briefly, a methanolic solution of ellagic acid (0.03 mM, 100 μL) was mixed with the analytical solution (100 μL) of the extract of *P. guajava*. Twenty microliters of the mixed solution was injected into the HPLC and analyzed under the same conditions mentioned above. The identity and purity of peak **1** in the HPLC chromatogram were examined by comparison of its UV spectral data with those of pure ellagic acid and peak **1** of the extract of *P. guajava*.

Identification of Compound 2 in the Extract of *C. depressa*. The dried stems (90 g) of *C. depressa* were ground by a mill (SCN-40A, Shibata, Tokyo, Japan), and 700 mL of 99% ethanol was added to the ground stems. The suspension was allowed to stand for 2 weeks at room temperature with occasional shaking. After filtration of the stems, the filtrate was evaporated under reduced pressure to give the ethanol extract (3.48 g). Twenty milligrams of the extract was separated by preparative HPLC under the following conditions: column, Daisopak ODS-120-AP (250 × 20 mm i.d.) (Daiso, Osaka, Japan); solvent, CH_3OH/H_2O/acetic acid (60:40:1); flow rate, 9.5 mL/min; detection, 254 nm. The peak eluted at 17 min was collected. After concentration, the residue was acetylated with acetic anhydride (1 mL) and pyridine (1 mL) at 25 °C for 1 h. Removal of the acetic anhydride and pyridine gave a mixture of the acetylated compounds including the acetate of compound **2**. These procedures were repeated 83 times. From 2.06 g of the extract, 20 mg of the crude acetate was obtained. The acetate was further purified by silica gel TLC (Merck 1.05744), which was developed with ethyl acetate/hexane = 1:1 to give the pure acetate of compound **2** (**2a**) (R_f = 0.3, 5.6 mg): HR-FABMS (m-NBA), (*m*/*z*) $[M]^+$ calcd for $C_{18}H_{14}O_8$ 358.0689, found 358.0706; ^{1}H NMR ($CDCl_3$) δ 13.80 (1H, s, OH-1), 8.24 (1H, d, J = 9.0 Hz, H-8), 7.11 (1H, d, J = 9.0 Hz, H-7), 6.29 (1H, d, J = 2.0 Hz, H-2), 6.19 (1H, d, J = 2.0 Hz, H-4), 3.87 (3H, s, OCH_3-3), 2.49 (3H, s, CH_3CO_2-5 or CH_3CO_2-6), 2.34 (3H, s, CH_3CO_2-5 or CH_3CO_2-6); ^{13}C NMR ($CDCl_3$) δ 180.4 (C-10), 167.4 ($CH_3\underline{C}O$), 167.2 ($CH_3\underline{C}O$), 166.2 (C-3), 164.7 (C-1), 145.8 (C-6), 142.0 (C-4a), 134.5 (C-5a), 128.8 (C-5), 124.5 (C-8), 118.9 (C-8a), 116.4 (C-7), 104.6 (C-1a), 95.6 (C-2), 90.3 (C-4), 55.6 (CH_3O), 20.8 ($\underline{C}H_3CO$), 20.6 ($\underline{C}H_3CO$). These assignments were based on the results from HMQC, HMBC, and NOESY experiments.

Identification of Compound 3 in the Extract of *H. chinense*. The dried stems (117 g) of *H. chinense* were ground by a mill (SCN-40A, Shibata, Tokyo, Japan), and 700 mL of 99% ethanol was added to the ground stems. The suspension was allowed to stand for 2 weeks at room temperature with occasional shaking. After filtration of the stems, the filtrate was evaporated under reduced pressure to give a brown oil (4.09 g). A part of the oil (2.88 g) was dissolved in methanol (100 mL) and H_2O (10 mL) and then partitioned with hexane (100 mL). The methanolic layer was evaporated to give 2.04 g of the residue. Twenty milligrams of the residue in methanol (200 μL) was injected into the preparative HPLC under the following conditions: column, Daisopak ODS-120-AP (250 × 20 mm i.d.); solvent, CH_3CN/H_2O/acetic acid (30:70:1); flow rate, 9.5 mL/min; detection, 254 nm. This purification was repeated 30 times. The peak at 20 min as retention time was collected, combined, and evaporated to give a 2.7 mg of solid. The solid was dissolved in a small amount of dimethyl sulfoxide and then injected into a recycle HPLC system. After six recycles, compound **3** showed only one peak. The peak was collected and evaporated to give the pure compound **3** (1.7 mg): HR-EIMS, (*m*/*z*) $[M]^+$ calcd for $C_{14}H_{10}O_5$ 258.0528, found 258.0549; EI-MS, *m*/*z* 258 $[M]^+$ (100%), 243 (45%), 215 (20%); ^{1}H NMR (DMSO-d_6) δ 8.14 (1H, d, J = 8.0 Hz, H-8), 7.77 (1H, br t, J = 7.6 Hz, H-6), 7.59 (1H, d, J = 8.4 Hz, H-5), 7.40 (1H, br t, J = 7.2 Hz, H-7), 7.27 (1H, br s, OH-3 or OH-4), 7.09 (1H, s, H-2), 6.62 (1H, br s, OH-3 or OH-4), 3.80 (3H, s, OCH_3-1).

Next, acetylation of compound **3** was carried out. To 0.6 mg of compound **3** were added acetic anhydride (0.5 mL) and pyridine (0.5 mL), and the solution was then allowed to stand for 1 h at room temperature. After removal of acetic anhydride and pyridine in vacuo, the residue was purified by silica gel TLC (ethyl acetate/hexane = 1:1) to give the diacetate of compound **3** (**3a**): IR (dry film), ν_{max} 1784, 1666, 1620 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.34 (1H, dd, J = 8.2 and 1.7 Hz, H-8), 7.73 (1H, s, H-2), 7.72 (1H, dt, J = 8.2 and 1.7 Hz, H-6), 7.47 (1H, dd, J = 8.6 and 1.0 Hz, H-5), 7.41 (1H, dt, J = 8.6 and 1.0 Hz, H-7), 3.95 (3H, s, OCH_3-1), 2.45 (3H, s, CH_3CO_2-3 or -4), 2.35 (3H, s, CH_3CO_2-3 or -4); NOE was observed between OCH_3-1 and H-2 in the NOE differential spectrum.

Comparison of DPPH Radical Scavenging Activity of Compound 3, Trolox, and Catechin. Each 200 μL of 1.5 mM methanol solutions of compound **3**, Trolox, and catechin was mixed well. Twelve microliters of the mixed solution was analyzed by HPLC under the following conditions: column, ODS-80Ts (150 × 4.6 mm i.d.); solvent A, 0.1% H_3PO_4; solvent B, CH_3CN; gradient system, linear gradient from 20% of solvent B to 60% of solvent B (60 min) and then 100% of solvent B (100 min); flow rate, 0.5 mL/min; detection, 254 nm. To the mixed solution was added 100 μL of a 3 mM DPPH methanolic solution. The mixture was allowed to stand for 3 min at 25 °C. An aliquot (14 μL) was analyzed under the above-mentioned conditions. This procedure was repeated three times, and HPLC analytical data were obtained in each case. The percent of the residual peak area to the initial area of each compound was calculated.

RESULTS AND DISCUSSION

Detection of Powerful Antiradical Compounds in the Extracts of *P. guajava*, *C. depressa*, and *H. chinense*. **Figures 1A**, **2A**, and **3A** show the analytical data of the total constituents of each plant extract, which were obtained by a gradient HPLC analysis. In these results, many kinds of constituents were observed from a lower hydrophobic region (short retention time) to a higher hydrophobic region (long retention time), which indicated that many fractionation steps were required for complete separation of all the constituents of the plant extracts. This classical stepwise fractionation approach is obviously thought to be very time-consuming for the identification of the antiradical compound from the extract and may produce an unsuccessful result due to the decomposition of the antiradical compounds during the repeated fractionation. On the other hand, **Figures 1B**, **2B**, and **3B** show the analytical reaction results of each extract by the addition of DPPH. The peak due to 1,1-diphenyl-2-picrylhydradine (DPPH-H), which was hydrogen-absorbed DPPH, was observed at 70.5 min as the retention time in **Figure 1B** and at 50.1 min in both **Figures 2B** and **3B**. In the HPLC data of *P. guajava* (**Figure 1**), the intensity of the peak at 36.8 min is typically decreased by the addition of DPPH (final concetration as added DPPH = 1.5 mM) when compared with that in the original data (panel A). This phenomenon indicates that this peak compound **1** is possibly the most active antiradical compound in the constituents of the extract of *P. guajava*. Next, panels A and B in **Figure 2** are the HPLC analytical data of the extract from *C. depressa* before and after the reaction with DPPH (final concentration as added DPPH = 1.0 mM), respectively. These data are rather more complicated than those of *P. guajava*; however, the intensity decrease was observed only in the peaks at 33.9 and 47.3 min in the HPLC data of the plant. The decrease in the peak at 33.9 min was apparent; therefore, this peak for compound **2** may be the main antiradically active compound in the extract of *C. depressa*. Finally, the analytical data for the extract of *H. chinense* are shown in **Figure 3**. These data are the most complicated among the three plant extracts. Comparison of each peak intensity between the original data (panel A) and the DPPH-reacted data (panel B, concentration of added DPPH = 1.7 mM) revealed that the decrease in the two peaks existed at 28.4 and 54.5 min, which were typically observed. These peak compounds should contribute to the strong antiradical activity of the extracts.

Identification of the Detected Antiradical Compounds. *Compound **1** of P. guajava.* By our simple analytical method, only one peak (**1**), which was observed at 36.8 min on the HPLC chromatogram, was selected as the peak corresponding to the powerful antiradical compound **1**. The UV spectrum of compound **1**, which was obtained by a DAD on our HPLC instrument, showed a very characteristic absorption pattern as shown in **Figure 4A**. Previously, we have isolated ellagic acid as a potent antioxidant from seashore plants (*13*). The UV spectrum of the obtained ellagic acid is very similar to that of peak compound **1**, indicating that **1** would be ellagic acid. An HPLC co-injection experiment with the extract and pure ellagic acid confirmed that compound **1** was ellagic acid (**Figure 5**).

*Compound **2** of C. depressa.* The UV spectrum for peak compound **2**, which was also obtained by a DAD, gave no other typical information except for the existence of a complicated conjugated moiety in compound **2** (**Figure 4B**). Therefore, further instrumental analyses, such as NMR and MS, were required to identify the structure of compound **2**. The isolation of compound **2** was achieved by direct separation of an ethanolic extract of *C. depressa* using a preparative HPLC as described under Materials and Methods. By the preparative HPLC, the peak mainly containing compound **2** was collected; however, compound **2** was found to be unstable for further purification as is. Therefore, compound **2** was purified and isolated by silica gel TLC as the diacetate form (**2a**). High-resolution FABMS analysis of **2a** indicated that its molecular formula was $C_{18}H_{14}O_8$. ^{13}C NMR data revealed the presence of a conjugated carbonyl carbon (δ 180.4) in addition to two acetyl carbonyl carbons (δ 167.4 and 167.2). Twelve carbon signals in the aromatic region, five or six signals of which were due to oxygenated carbons, were observed. These results indicated that **2a** was an oxygenated xanthone derivative. From the ^1H NMR data, the substitution pattern of the xanthone ring was deduced. Typically a low-field-shifted proton signal at 13.8 ppm indicated that the signal was due to a hydrogen-bonded hydroxyl proton. An aromatic hydrogen signal, which has an ortho-coupled proton (J = 9.0 Hz), was also low-field-shifted to 8.24 ppm. These data indicated that a hydroxyl group and a proton were adjacent to the carbonyl group of the xanthone. Two other aromatic protons, which were meta-coupled to each other (J = 2.0 Hz), were correlated with a methoxyl signal at 3.87 ppm in the NOESY data of **2a**. From these data, compound **2a** was determined to be 1-hydroxy-3-methyoxy-5,6-diacetoxyxanthone, as depicted by structure **2a** in **Figure 5**. Therefore, the antiradical compound **2** should be 1,5,6-trihydroxy-3-methoxy-xanthone (**2**), which has been already identified in *Hypericum androsenum* by Niesen and Arendes (*14*).

*Compound **3** of H. chinense.* To identify the compound corresponding to the peak at 28.4 min, the isolation of peak compound **3** was carried out. After removal of the nonpolar substances from the ethanol extract of *H. chinense* by a solvent partition technique using hexane, the residual fraction was directly separated by preparative HPLC. Compound **3** was finally purified by recycle HPLC to pure form. The molecular formula of **3** was determined to be $C_{14}H_{10}O_5$, which was deduced by HR-EIMS. In its ^1H NMR data, five aromatic proton signals were observed at 8.14, 7.77, 7.59, 7.40, and 7.09 ppm.

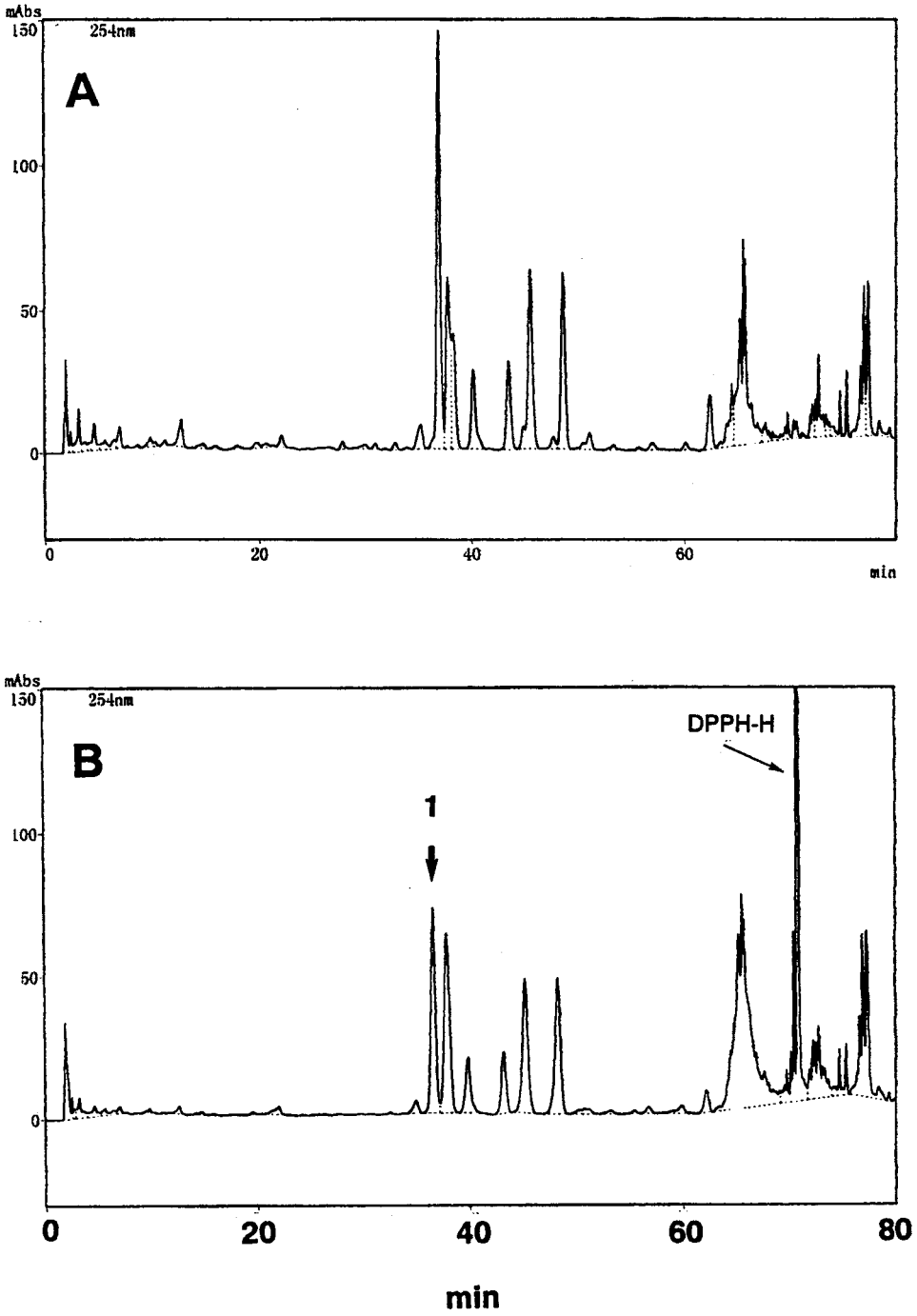

Figure 1. HPLC analytical data for the extract from *P. guajava*: (A) intact extract data; (B) DPPH (1.5 mM)-added extract. Arrow indicates the peak (retention time at 36.8 min) for the reacted compound with DPPH.

Other proton signals were assignable to two phenolic hydroxyls (δ 7.27 and 6.62) and a phenolic methoxyl group (δ 3.80). In addition, a typical absorption of a highly conjugated carbonyl group was observed at 1660 cm^{-1} in the IR spectrum of the acetate of **3** (**3a**). Thus, compound **3** should have a xanthone structure similar to that of compound **2**. The substitution pattern on the mother ring was next elucidated. From the proton coupling network, four aromatic proton signals at 8.14, 7.77, 7.59, and 7.40 ppm were coupled to each other, indicating that one of the aromatic parts of the xanthone has no substituent. Therefore, two hydroxyl groups and one methoxyl group should be substituted to the other aromatic ring. It should be noted that the remaining proton signal was not low-field-shifted (δ 7.09). This suggested that the adjacent position of a carbonyl group on the aromatic ring was substituted by some functional group other than a proton. However, a very low-field-shifted hydrogen signal, which indicated the presence of a hydrogen-bonded hydroxyl group adjacent to the carbonyl group, was also not observed, indicating that the position adjacent to the carbonyl group should be occupied by the methoxyl group. The methoxyl group presented an NOE enhancement to the proton signal at the 2-position (δ 7.73) on the acetate of compound **3** (**3a**). From these data, compound **3** was determined to be 3,4-dihydroxy-1-methoxyxanthone, which is a newly identified compound to the best of our knowledge.

Comparison of Antiradical Efficiency of Compound 3 with That of Known Powerful Antiradical Compounds Catechin and Trolox. A comparison of the DPPH radical scavenging

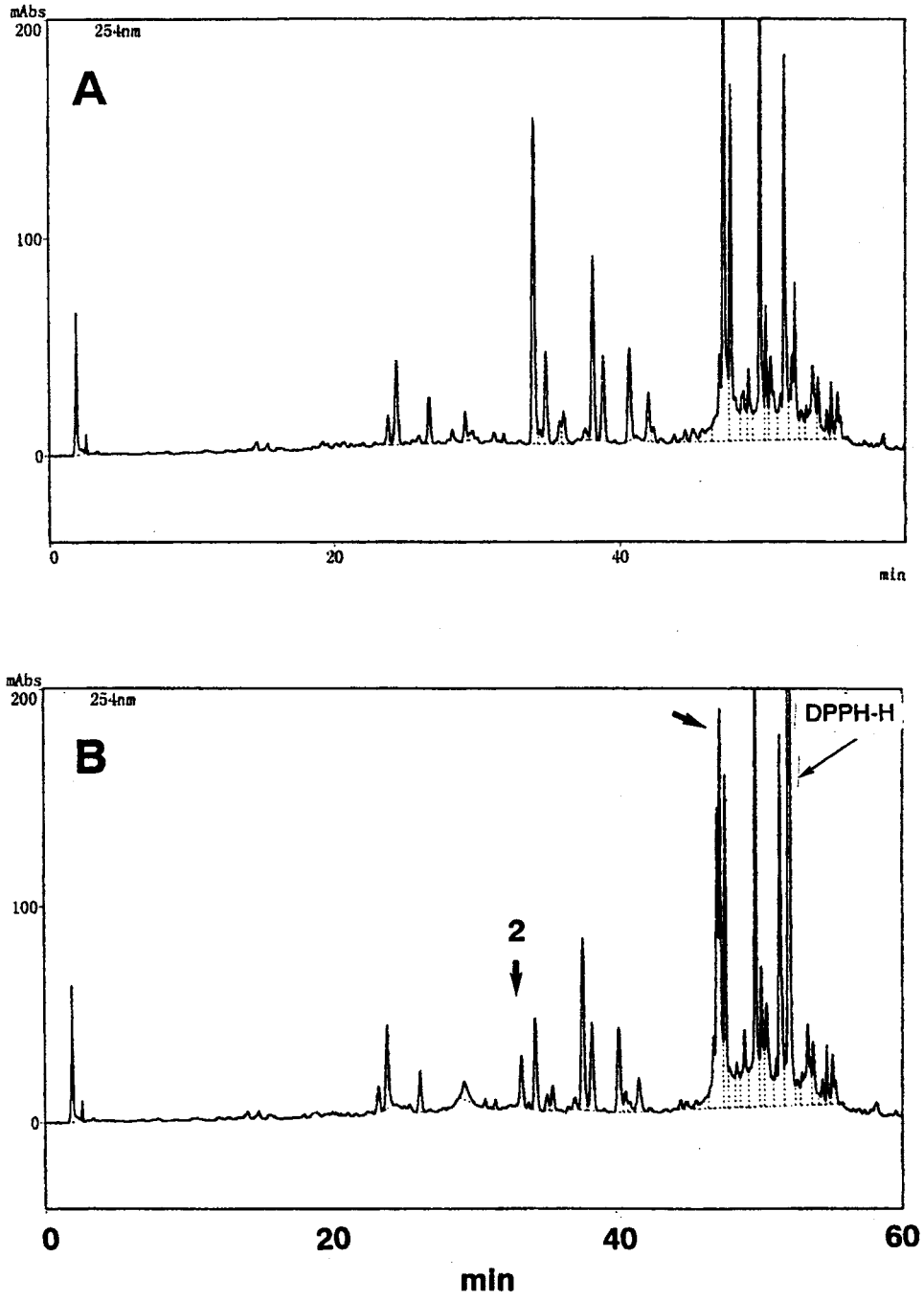

Figure 2. HPLC analytical data for the extract from *C. depressa*: (A) intact extract data; (B) DPPH (1.0 mM)-added extract. Arrow indicates the peak (retention times at 33.9 and 47.3 min) for the reacted compound with DPPH.

ability of a compound can be performed as an application of our developed method. The radical scavenging abilities of the newly identified compound **3** and known antioxidants, catechin and Trolox, were compared. **Figure 6** shows the peak decrease of each compound by the consecutive addition of DPPH to the solution consisting of equal concentration of the three compounds. After the addition of one portion (100 μL) of the DPPH solution (3.0 mM), the peak corresponding to Trolox decreased to 50% of the initial peak intensity. The other peak showed almost no decrease in intensity. The addition of one more portion (100 μL) of the DPPH solution showed that Trolox had almost completely disappeared from the HPLC data. After the addition of a total of three portions of the DPPH solution, a clear decrease was observed between the peaks of catechin and compound **3**. From the decrease ratio, the radical-scavenging ability of compound **3** was slightly stronger than that of catechin. Thus, the order of the radical scavenging ability is Trolox $\gg$ compound **3** > catechin. Trolox is a vitamin E analogue and has a very strong radical scavenging ability with the reaction rate of 2.23×10^8 M^{-1} s^{-1} against the CCl_3O_2 radical (*15*). Although the radical scavenging rate of catechin (6.1×10^6 M^{-1} s^{-1}) (*15*) is much weaker than that of Trolox, catechin and related compounds are now recognized as some of the strongest antioxidants in food and living systems. Therefore, practical antioxidant activity of **3** should be desired in these systems.

In conclusion, our present method, which is based on the feature of an antioxidant reaction, is very simple and requires

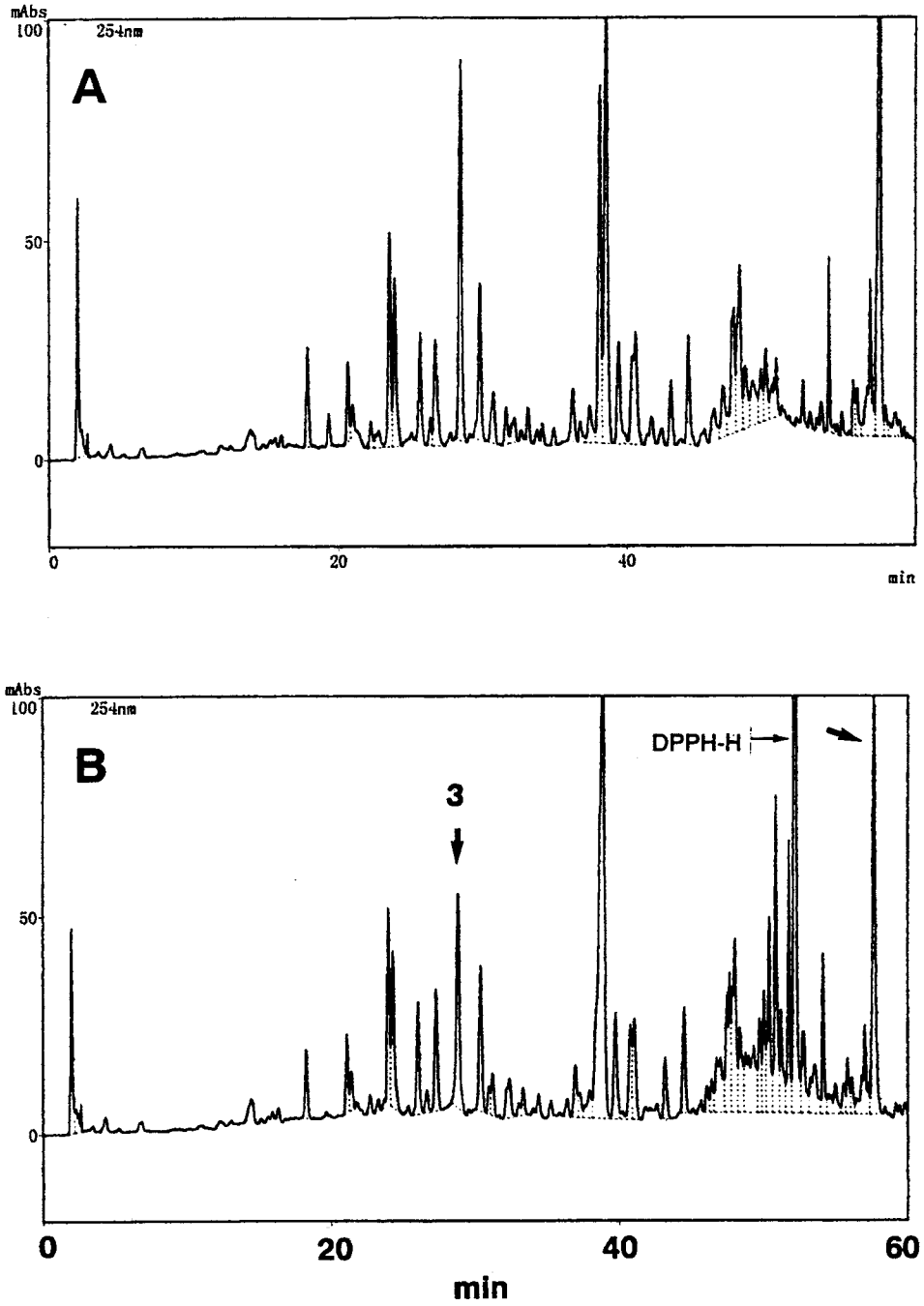

Figure 3. HPLC analytical data for the extract from *H. chinense*: (A) intact extract data; (B) DPPH (1.7 mM)-added extract. Arrow indicates the peak (retention times at 28.4 and 54.5 min) for the reacted compound with DPPH.

only a conventional HPLC instrument without special equipment for detection and reaction systems unlike other on-line methods. We used a DAD as the detection system, which affords UV spectral data of the antiradical compounds without isolation; however, various advanced detection systems, such as LC-MS, LC-IR, and LC-NMR, have been recently developed. This method could possibly use these new detectors as is and can be expected to provide more structural information for each peak compound when these systems are employed.

In addition, our method can select only the most powerful antiradical compound against DPPH from a mixture of a large variety of compounds. It should be noted that all of the antioxidants could not be detected by the reaction with DPPH and various assay methods have been used for antioxidant detection so far (*16*). Some radical reagents used in the assay methods, such as ABTS(2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) or chemically generated peroxyl radical, could be applicable as a substitute of DPPH in this method. Consideration of the results obtained using the different characteristic radical reagents from DPPH would enable more precise evaluation of the antioxidant ability of constituents in a raw extract. At least, the present method will avoid the time-consuming repetition of fractionation and the subsequent activity measurement steps in an isolation study.

Finally, this method is easily applicable to the measurement and comparison of the antiradical efficiency of a compound. Although this method does not provide accurate quantitative information, such as reaction rate, for the activity of a compound, this procedure is very easy to operate with a

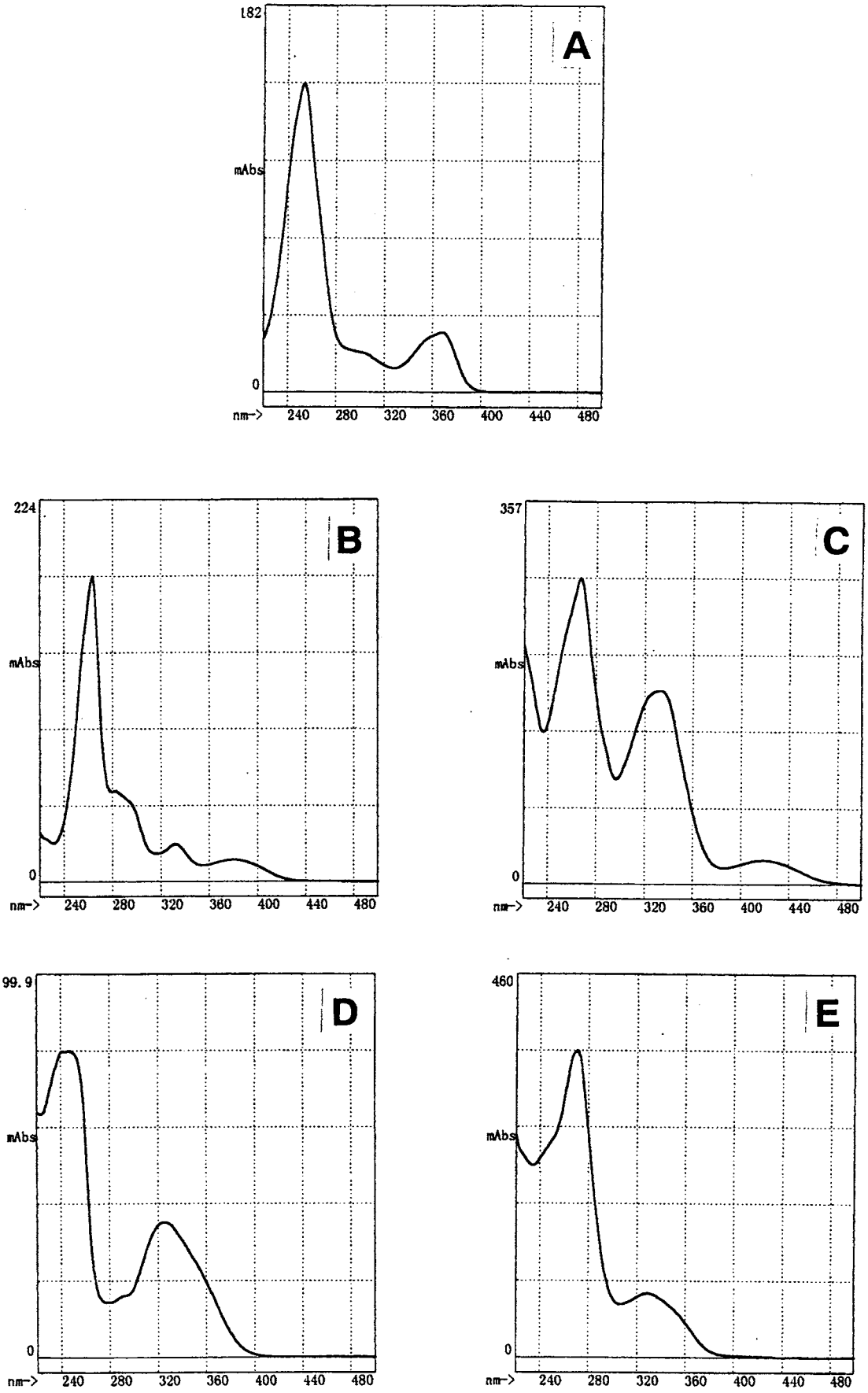

Figure 4. UV spectra of the intensity-decreased peaks on the HPLC data: (A) 36.8 min peak on the HPLC data of *P. guajava*; (B) 33.9 min peak on the HPLC data of *C. depressa*; (C) 47.3 min peak on the HPLC data of *C. depressa*; (D) 28.4 min peak on the HPLC data of *H. chinense*; (E) 54.5 min peak on the HPLC data of *H. chinense*.

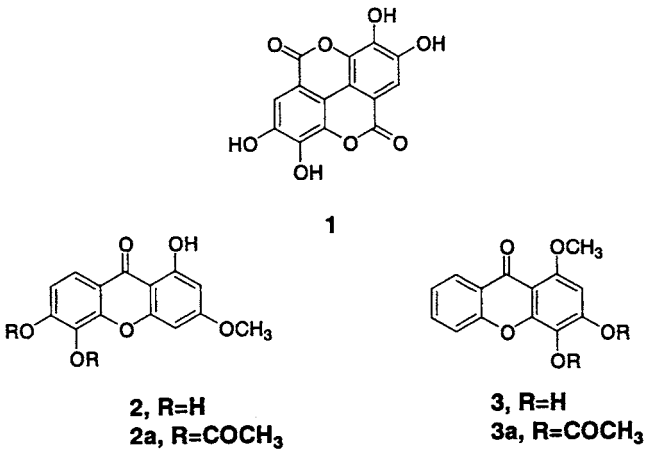

Figure 5. Chemical structures for compounds **1**–**3** identified as powerful antiradical substances.

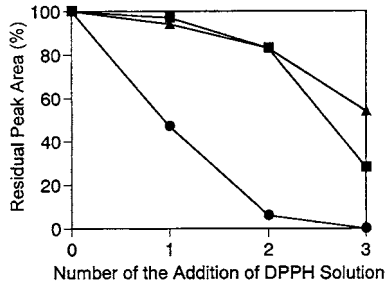

Figure 6. Comparison of the antiradical activities of Trolox (●), catechin (▲), and compound **3** (■) by gradual addition of 3.0 mM DPPH solution. Initial concentration for Trolox, catechin, and compound **3** was 1.5 mM; one portion of the 3.0 mM DPPH solution was 100 μL.

simple HPLC to define the activity order of the compounds examined.

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