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Low-density lipoprotein-antioxidant flavonoids and a phenolic ester from *Plectranthus hadiensis* var. *tomentosus*

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Abstract

To investigate the effects of extraction solvents and drying methods on *Plectranthus hadiensis* var. *tomentosus* quality, eight compounds were isolated and the content of active compounds with their antioxidant activities were compared. Compounds **1** and **2** were known antioxidants, whereas the low-density lipoprotein (LDL)-antioxidant activities of compounds **3**, **5**, **6**, and **7** are reported for the first time, with IC_{50} values of 2.5, 3.8, 22.8, and 53.7 μM, respectively. Our analysis of 30–95% ethanol extracts from freeze- and air-dried leaves and stems revealed a relationship between extract composition and antioxidant activity. The 95% ethanol extracts of freeze-dried stems (FDS) exhibited highest phenolic and flavonoid content, which were 1.40 and 2.67 times, respectively, greater than those of air-dried stems (ADS), and very high LDL-antioxidant and DPPH radical scavenging activities, which may have resulted from the phenolic ester rosmarinic acid (**2**), a major component of FDS extracts and potent antioxidant. In contrast, the 95% ethanol extracts of ADS exhibited relatively low antioxidant activity, possibly owing to the low antioxidant activity of the main components ayanin (**7**) and (+)-plectranthone (**8**). These results are important for the development of *P. hadiensis* var. *tomentosus* as an effective natural antioxidant material.

Keywords: Antioxidant, Flavonoid, Phenolic compounds, *Plectranthus hadiensis* var. *tomentosus*, Rosmarinic acid

Introduction

The excess production of oxidants, such as reactive oxygen species, and imbalances between antioxidative defence systems and active oxygen molecules can induce oxidative stress, and such oxidative stress has been linked to the pathogenesis of various chronic diseases, including cardiovascular disease [1], atherosclerosis [2], cancer [3], and inflammatory disorders [4]. Accordingly, the application of antioxidants for the treatment of various pathological diseases has gained the attention of the food, cosmetic, and pharmaceutical industries, as well as of the research community [5]. Furthermore, many natural antioxidants are derived from herbs, spices, tea, and fruits [6], and the antioxidant effects of such products are correlated with their polyphenol content [7, 8].

Plants of the genus *Plectranthus* (family Lamiaceae) are used for the treatment of digestive problems, skin disease, infection/fever, pain, and allergies, with a wide diversity of ethnobotanical uses [9]. The *Plectranthus* plants are enriched in phenolics and essential oils, including terpenoids [10]. However, the phytochemical constituents and biological roles of *P. hadiensis* var. *tomentosus* (Benth. ex E.Mey.) Codd (PHT), a succulent-like perennial herb found in South Africa and Asia, including South Korea, have been poorly documented. In the present study, we isolated eight compounds from the aerial parts of PHT, and analysed their antioxidant activity by evaluating their ability to inhibit Cu^{2+}-mediated low-density lipoprotein (LDL) oxidation and apoB-100 fragmentation and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals.

Both hot air- and freeze-drying are commonly used for preserving plant products and air-drying can drastically

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reduce the quality of the original material [11]. In addition, the optimized processes of the extraction method using solvents is very important, so that the maximum quantities of active compounds can be obtained for their development and commercialization. Therefore, we evaluated the antioxidant abilities of various ethanol (EtOH) extracts from both freeze- and air-dried PHT leaves and stems.

Materials and methods

Plant material

Aerial parts of PHT were cultivated and collected on August in Daejeon, Korea, and were identified by Dr. Su-Young Kim (Plant Resources Division, National Institute of Biological Resources, Incheon, Korea). A voucher specimen (No. CNU13101) was deposited in the herbarium of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Instruments and chemicals

All purifications were monitored on commercially available glass-backed, pre-coated thin-layer chromatography plates (Merck, Darmstadt, Germany) and were visualized under UV at 254 nm and 365 nm or stained with *p*-anisaldehyde solution. Medium pressure liquid chromatography (MPLC) was conducted using BiotageIsolera (Biotage AB, Uppsala, Sweden) and a flash SiO_2 column (SNAP Cartridge Silica-gel FLASH 40 + M, 4.0 × 15.0 cm; Biotage). Column chromatography was performed using Diaion HP-20 resin (250–850 μm, Mitsubishi Chemical Co., Tokyo, Japan), silica gel (200–300 mesh, Merck, Germany), octadecyl silica-gel (ODS; ODS-A, 12 nm, S-150 μm; Merck), Sephadex LH-20 (25–100 μm; GE Healthcare Biosciences, Uppsala, Sweden), and Sep-Pak C18 (Waters Co., Milford, MA, USA). All the solvents used for extraction and isolation were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction and isolation

Fresh aerial parts of PHT (3.5 kg) were ground and extracted with 20 L 95% EtOH for 72 h at room temperature, and the 95% EtOH extract was concentrated *in vacuo* to yield a brown residue (48.8 g). The residue was suspended in 10% aqueous methanol (MeOH) and partitioned with *n*-hexane (1000 mL × 3) and chloroform ($CHCl_3$) (1000 mL × 3), successively.

The *n*-hexane layer (7.5 g) was subjected to medium pressure liquid chromatography (MPLC) using a SiO_2 column (4.0 × 15.0 cm) with an *n*-hexane–ethyl acetate (EtOAc) gradient of 1:0 to 0:1 (v/v) and a flow rate 20 mL/min, in order to obtain eight fractions (H1–H8). Fraction H5 (*n*-hexane–EtOAc, 1:1, 1.2 g) was further separated using another SiO_2 column (2.0 × 27.0 cm), with *n*-hexane–EtOAc (7:1, 5:1, 4:1, 2:1, and 1:2, v/v, 250 mL, each), in order to obtain four sub-fractions (H5-1–H5-4). Sub-fraction H5-2 (4:1, 650 mg) was purified using Sephadex LH-20 column (1.5 × 60.0 cm) eluting with $CHCl_3$–MeOH (1:20, v/v) and further purified using SiO_2 column (1.0 × 26.5 cm) with *n*-hexane–EtOAc (7:1, 6:1, 4:1, v/v, 40 mL, each) to obtain compound **8** (127.4 mg) and H5-2-2-3 (4:1, 40.0 mg, which was purified using ODS column (1.0 × 26.0 cm) with $CHCl_3$–MeOH–H_2O (5:3:1, v/v/v) to obtain compound **4** (9.5 mg). Fraction H6 (4:6, 0.7 g) was further separated using SiO_2 column (2.0 × 27.5 cm) with *n*-hexane–EtOAc (7:1, 5:1, 4:1, 2:1, and 1:2, v/v, 250 mL, each), to obtain five sub-fractions (H6-1–H6-5), and sub-fraction H6-4 (2:1, 323 mg) was purified using Sephadex LH-20 column (1.5 × 64.0 cm) with $CHCl_3$–MeOH (1:100, v/v) to obtain compound **7** (42.2 mg).

The $CHCl_3$ layer (2.1 g) was subjected to SiO_2 column (3.5 × 26.0 cm) with *n*-hexane–EtOAc (5:1, 4:1, 3:1, 2:1, 1:1, 1:3, and 1:5, v/v, 500 mL, each), in order to obtain seven fractions (C1–C7). Fraction C5 (2:1, 188 mg) was further separated using SiO_2 column (2.0 × 30.0 cm) with $CHCl_3$–MeOH (300:1, 200:1, 100:1, and 50:1, v/v, 200 mL, each) to obtain four sub-fractions (C5-1–C5-4), and sub-fraction C5-4 (100:1–50:1, 138 mg) was purified using Sephadex LH-20 column (1.5 × 62.0 cm) with $CHCl_3$–MeOH (1:10, v/v) to obtain compound **5** (6.0 mg). Fraction C6 (1:1–1:3, 306 mg) was further separated using ODS column (2.2 × 28.5 cm) with $CHCl_3$–MeOH (1:1, 2:3, 0:1, v/v, 220 mL, each) to obtain six sub-fractions (C6-1–C6-6). Sub-fraction C6-2 (1:1, 98 mg) was separated using Sephadex LH-20 column (1.5 × 60.0 cm) with MeOH–H_2O (1:1, v/v) and subsequently purified using preparative TLC (RP-18, 20.0 × 10.0 cm; Merck) with MeOH–H_2O (4:1, v/v) to obtain compound **3** (12.8 mg). Continually, sub-fraction C6-4 (1:1, 65 mg) was purified using SiO_2 column (1.0 × 28.0 cm) with $CHCl_3$–MeOH (75:1 to 1:1, v/v) to obtain compound **6** (30.4 mg).

The 10% aqueous MeOH layer (24.9 g) was subjected to Diaion HP-20 column (70.0 × 40.0 cm) with MeOH–H_2O (0:1, 1:4, 2:3, 3:2, 4:1, and 1:0, v/v, 3.0 L, each), in order to obtain six fractions (M1–M6). Fraction M4 (4:1, 1.5 g) was further separated using ODS column (3.0 × 30.0 cm) with MeOH–H_2O (1:3, 1:2, and 3:1, v/v, 400 mL, each) to obtain five sub-fractions (M4-1–C4-5). Sub-fraction M4-2 (1:3, 300 mg) was purified using Sephadex LH-20 column (1.0 × 63.0 cm) with MeOH–H_2O (1:2, v/v) to obtain compound **2** (72.4 mg), and sub-fraction M4-3 (MeOH–H_2O. 1:2–1:1, 900 mg) was further separated using Sephadex LH-20 column (1.0 × 45.0 cm) with MeOH–H_2O (1:1, v/v) to obtain compound **1** (50.7 mg).

Analysis of crude leaf and stem extracts

Preparation of crude extracts

Freeze- and air-drying of PHT leaves and stems were performed using a freeze dryer (Alpha 1–4 LD plus; Marin Christ, Osterode, Germany) and an air dryer (Daihan Labtech Co., Namyangju, Korea) at 60 °C, respectively. The freeze- and air-dried leaves and stems (FDL, FDS, ADL, and ADS, respectively) were extracted with 20 mL of 30%, 50%, 70%, and 95% EtOH for 48 h at room temperature, and the extracts were filtered and then dehydrated using a speed-vacuum evaporation system (Thermo Savant, NY, USA). The resulting samples were then analysed to evaluate their major components, total phenolic and flavonoid content, and LDL-antioxidant and DPPH radical scavenging activities.

Determination of total phenolic and flavonoid content

The total phenolic content of the extracts were measured using a modified version of the Folin–Ciocalteu method [12] and were expressed as mg catechin equivalent (CE) per g extract. The total flavonoid content of the extracts were measured using a modified colorimetric method [13] and were expressed as mg CE per g extract.

HPLC analysis and main component quantification

In the present study, the characteristic variations in the 30–95% EtOH extracts of FDL, FDS, ADL, and ADS from PHT were determined using a Shimadzu HPLC system (Shimadzu Co., Tokyo, Japan), equipped with a binary pump delivery system, a photodiode array detector (PDA), and an auto-sampler, and a Brownlee SPP C18 column (4.6 × 50 mm, 2.7 μm; PerkinElmer, Inc., Waltham, MA, USA). The injection volume was 5 μL, and 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B) were used as the mobile phases. The linear gradient elution program was as follows: 5–40% B at 0–15.0 min, 40–100% B at 15.0–20.0 min, 100–5% B at 20.0–22.5 min, and 5% B at 22.5–25.0 min. The flow rate was 1.8 mL/min, and the absorbance was 254 nm.

After evaluating the characteristic variations of the eight compounds in the HPLC profiles of the different extracts, compounds **1**, **2**, and **7** were selected for quantitative analysis. Compounds **1**, **2**, and **7** that were isolated from the crude extracts of PHT were used as standards, and after being filtered through a 0.45-μm membrane filter (Whatman, Wallingford, UK), calibration curves were generated from serial dilutions of the three individual compounds by plotting the concentration of each sample against its HPLC peak area obtaining the slope (s), standard deviation (σ), and correlation coefficient (R^2) of each curve. Subsequently, the three individual compounds were quantified (mg/g extract) in the FDS, FDS, ADL, and ADS extracts using the regression equations of the corresponding standard curves, and validation of the quantification method was conducted, according to the International Conference of Harmonization (ICH)-Q2 guidelines [14].

DPPH radical scavenging activity

The DPPH radical scavenging activity of the samples was determined by measuring the decolorization of DPPH from the trapping of its unpaired electron, using the procedure described by Kang et al. [15], with slight modifications. Briefly, 190 μL fresh DPPH radical solution in MeOH (150 μM) was added to 10 μL of each sample and incubated for 40 min at room temperature. The amount of DPPH radical remaining was then determined by measuring the solution's absorbance at 517 nm with a model 680 Microplate reader (Bio-Rad, Inc., Hercules, CA, USA). The antioxidant butylated hydroxytoluene (BHT) and L-ascorbic acid were used as positive controls The DPPH radical scavenging activity was expressed as the percent of starting DPPH radical that was scavenged: DPPH radical scavenging activity (%) = 100% × (1 − absorbance of sample/absorbance of control).

Inhibition of Cu^{2+}-induced LDL oxidation

Blood from healthy volunteers was obtained from the Korean Red Cross Blood Center, Daejeon according to the Guidelines of Blood Donation Program for Research. After the plasma was separated by centrifuging the whole blood at low speed, EDTA (0.1%), NaN_3 (0.05%), and phenylmethylsulfonyl fluoride (0.015%) were added to the plasma, in order to prevent lipoprotein modification. The LDL was isolated from the plasma using discontinuous density gradient ultracentrifugation, as described previously [16].

TBARS assays were performed as described previously [17], with minor modifications. Briefly, 250 μL LDL solution (120 μg of protein in PBS) was supplemented with 10 μM $CuSO_4$ as an oxidation initiator, and oxidation reactions were performed in screw-capped 5 mL glass vials at 37 °C with or without test samples. After 4 h of incubation, the reactions were terminated by adding 1 mL 20% (v/v) trichloroacetic acid, and following precipitation, the mixtures were supplemented with 1 mL 0.67% (v/v) *tert*-butyl alcohol in 0.05 N NaOH, vortexed, heated for 5 min at 95 °C, cooled on ice, and centrifuged for 2 min at 1000×*g*. Subsequently, the optical density of malondialdehyde (MDA), which was produced by LDL oxidation, was measured at 532 nm. BHT and L-ascorbic acid were used as positive controls in this assay, and calibration was performed using a MDA standard that was prepared from tetramethoxypropane [malondialdehyde bis (dimethyl acetal)].

The Cu^{2+}-mediated oxidation of LDL oxidation causes fragmentation of apoB-100. Therefore, we evaluated the ability of isolated compounds to inhibit LDL oxidation by measuring fragmentation of apoB-100 using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), as described previously [18]. After Cu^{2+}-induced oxidation of LDL, the reaction mixtures were denatured with 3% SDS, 10% glycerol, and 2 M DL-dithiothreitol at 95 °C for 5 min, and SDS-PAGE (5%) was performed to detect the apoB-100 fragmentation at 75 V for 150 min. After electrophoresis, the gel was dried and stained with Coomassie Brilliant Blue R250, and the density (AU/mm^2) of each apoB-100 band was measured using a GS-800 densitometer (Bio-Rad) with Bio-Rad Quantity One-4.4.2 software. BHT was used as a positive control.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Significant differences among the groups were assessed by one-way analysis of variance (ANOVA) and Student's *t*-test, using JMP software (SAS Institute Inc., Cary, NC, USA), and p value < 0.05 was considered significant.

Results and discussion

Isolation and identification of compounds 1–8

The 95% EtOH extract of fresh aerial parts of PHT was fractionated using *n*-hexane, $CHCl_3$, and 10% aqueous MeOH. The all three fractions were used for further isolation of active metabolites using repeated SiO_2, ODS, and Sephadex LH-20 column chromatographies: two sesquiterpenes (**4** and **8**) and one flavonoid (**7**) were isolated from the *n*-hexane fraction; three flavonoids (**3**, **5**, and **6**) were isolated from the $CHCl_3$ fraction; and one flavonoid (**1**) and one phenolic acid (**2**) were isolated from the 10% aqueous MeOH fraction (Fig. 1). Furthermore, the chemical structures of compounds **1**–**8** were identified as luteolin 7-*O*-glucuronide (**1**) [19], rosmarinic acid (**2**) [20], chrysosplenol D (**3**) [21], desacetyl plectranthone (**4**) [22], quercetin 3, 7-dimethyl ether (**5**) [23], casticin (**6**) [21], ayanin (**7**) [23], and (+)-plectranthone (**8**), the enantiomer of (−)-plectranthone [22], based on ^1H and ^{13}C NMR and ESI–MS data, and confirmed through comparison with published spectroscopic data (Fig. 1 and Additional file 1). This is the first report on the isolation of these eight compounds from PHT.

Antioxidant activities of compounds 1–8

The 95% EtOH extract of fresh aerial parts of PHT exhibited potent LDL-antioxidant activity (43.7 ± 0.6% inhibition at 20 μg/mL) and the *n*-hexane, $CHCl_3$, and 10% aqueous MeOH fractions exhibited LDL-antioxidant activities with 18.9 ± 1.8%, 94.6 ± 1.1%, and 23.5 ± 0.2% inhibition at 20 μg/mL, respectively. In addition, compounds **1**–**3** and **5**–**7** exhibited potent LDL-antioxidant activities in the TBARS assays with IC_{50} values of 2.8, 1.5, 2.5, 3.8, 22.8, and 53.7 μM, respectively, whereas the two

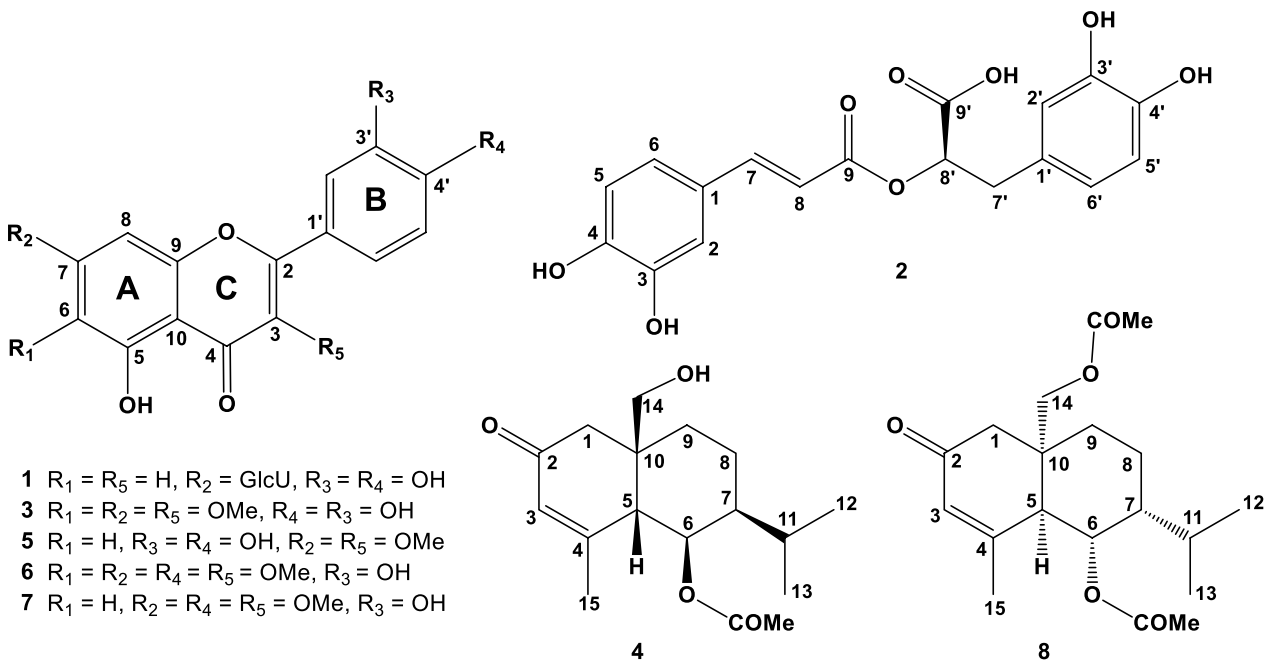

Fig. 1 Chemical structures of compounds **1**–**8** isolated from the leaves and stems of PHT

sesquiterpenes (compounds **4** and **8**) were not active, and the IC_{50} values of the positive control, BHT and L-ascorbic acid were 1.9 and 60.2 μM, respectively (Table 1). In the present study, the LDL-antioxidant activities of flavonoids **3**, **5**, **6**, and **7** are reported for the first time and the activities of flavonoids **3** and **5** were much higher than that of L-ascorbic acid.

Typically, LDL-antioxidant activities of phenolics and flavonoids are highly dependent on the configuration, position, and total number of hydroxyl groups [24–26], and indeed, compound **2**, which 3,4-dihydroxy, 3′,4′-dihydroxy, exhibited the highest LDL-antioxidant activity and is already known for its LDL-antioxidant activity [27, 28]. Furthermore, when we delineated the LDL-antioxidant activities of the flavonoids **1**, **3**, and **5**–**7** according to their structure–activity relationships. (i) a 3′,4′-catechol structure in the B-ring of the flavonoid skeleton strongly enhanced the inhibition of LDL-oxidation [29, 30]; (ii) benzene-ring methoxyl groups of phenols, especially A and B-ring methoxyl groups of the flavonol skeleton, decreased LDL-antioxidant activity, as was observed for compounds **1** (IC_{50} = 2.8 μM), **3** (IC_{50} = 2.5 μM), **5** (IC_{50} = 3.8 μM), **6** (IC_{50} = 22.8 μM), and **7** (IC_{50} = 53.7 μM); and (iii) an A-ring glycosyl group of the flavone skeleton retained or slightly enhanced the antioxidant activity, as indicated by comparing the activities of compounds **1** and **5**.

The inhibition of the oxidative process of compounds **1**–**3** and **5**–**7** were evaluated also by the fragmentation of apoB-100 through analysis via SDS-PAGE. We observed apoB-100 band for native LDL (120 μg/mL in PBS) that had been incubated without 5 μM $CuSO_4$ (i.e., non-oxidized), but the band was completely absent, when the LDL was incubated with 5 μM $CuSO_4$. We also observed that treatment of the native LDL with 5 μM of compounds **1**–**3** and **5** inhibited the Cu^{2+}-induced fragmentation of apoB-100 by 56.3%, 37.7%, 77.1%, and 84.6%, respectively, and the positive control (BHT) inhibited the fragmentation of apoB-100 by 54.0%. In this result, compounds **1**, **3**, and **5** exhibited significantly more activity than compound **2** (Fig. 2). The B-ring hydroxyl groups and position of the C-ring methoxyl group on carbon 3 were critical for the protection of apoB-100 fragmentation from Cu^{2+}-induced oxidation, as shown by compounds **3** and **5**. However, the effect was inconsistent, since compound **2** failed to exhibit the same protection in either the apoB-100 or TBARS assays. TBARS assay is a good method to measure the amount of oxidized lipid present in a medium; MDA, formed from the breakdown of polyunsaturated fatty acids by lipid hydroperoxidation, reacted with thiobarbituric acid to form a red chromophore and the TBARS was detected at 532 nm [17]. On the other hand, the Cu^{2+}-induced fragmentation of apoB-100 may result from derivatization of apoB lysine residues by peroxidation products or breakdown products, leading to a net increase of negative charge [18]. The reaction is generally inhibited by radical scavengers, such as β-mercaptoethanol, BHT, and probucol [31]. Thus, the results of some methods may not indicate the same effects on Cu^{2+}-induced oxidation of LDL.

Table 1 Antioxidant activity of compounds 1–8 from PHT

Compounds	LDL oxidation inhibition (IC_{50}, μM)[a]	DPPH radical scavenging (EC_{50}, μM)[b]
Luteolin 7-*O*-glucuronide (**1**)	2.8 ± 0.1	26.2 ± 0.2
Rosmarinic acid (**2**)	1.5 ± 0.1	19.0 ± 0.7
Chrysosplenol D (**3**)	2.5 ± 0.1	48.3 ± 0.1
Desacetyl plectranthone (**4**)	NI	NI
Quercetin 3,7-dimethyl ether (**5**)	3.8 ± 0.1	31.2 ± 0.1
Casticin (**6**)	22.8 ± 0.4	> 100
Ayanin (**7**)	53.7 ± 0.1	> 100
(+)-Plectranthone (**8**)	NI	NI
BHT	1.9 ± 0.4	> 100
L-Ascorbic acid	60.2 ± 1.3	28.1 ± 0.4

Values indicate mean ± SD

NI not inhibited

[a] IC_{50} values indicate the concentration (μM) of sample that caused 50% inhibition of Cu^{2+}-mediated LDL oxidation

[b] EC_{50} values indicate the concentration (μM) of sample that scavenged 50% of the DPPH radical

Typically, DPPH radical scavenging effects of phenolics and flavonoids are highly dependent on the configuration, position, and total number of hydroxyl groups [24, 25], and indeed, compound **2**, which 3,4-dihydroxy, 3′,4′-dihydroxy, exhibited the highest DPPH radical scavenging effects. Furthermore, when we delineated the DPPH radical scavenging effects of the flavonoids **1**, **3**, and **5**–**7** according to their structure–activity

The radical-scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O^{2-}), the hydroxyl radical (OH^-), or the peroxyl radical (ROO^-), is measured to test the ability of natural products to act as free radical scavengers or hydrogen donors. Of these methods, DPPH radical scavenging method is widely used to measure antioxidant capacity as a rapid, simple, and inexpensive method [32]. The phenolic compounds **1**–**3** and **5** were more effective than an antioxidant, BHT at radical scavenging with EC_{50} values as 26.2, 19.0, 48.3, and 31.2 μM, respectively (Table 1), whereas compounds **4** and **6**–**8** failed to exhibit DPPH radical scavenging activity. In this assay, L-ascorbic acid, a positive control, exhibited an EC_{50} of 28.1 μM.

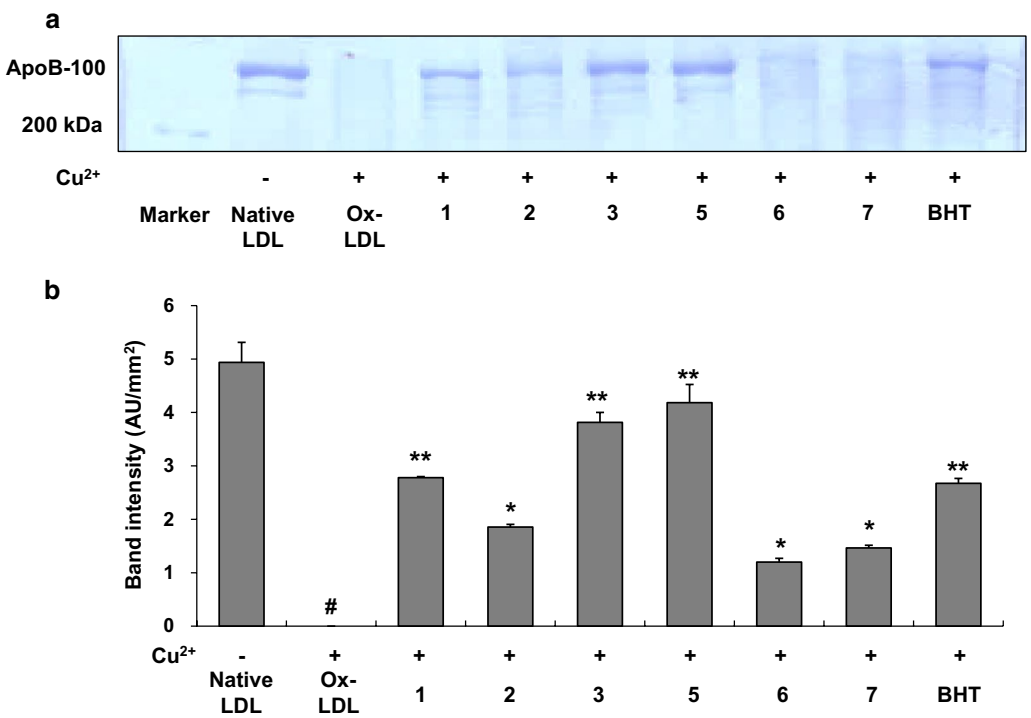

Fig. 2 Antioxidant effects of compounds **1**, **2**, **3**, **5**, **6**, and **7** from PHT on Cu^{2+}-mediated apoB-100 fragmentation. **a** The fragmentation of apoB-100 through analysis via SDS-PAGE. Five μM of each sample was run on an SDS-PAGE and BHT (2 μM) was used as a positive control. **b** Intensities of the apoB-100 bands are indicated as mean optical densities (absorbance units/mm^2) ± SD values of three independent experiments. $^{\#}p < 0.01$ vs. native LDL; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ox-LDL using the Student's *t*-test

relationships. (i) a 3′,4′-catechol structure in the B-ring of the flavonoid skeleton strongly enhanced the inhibition of DPPH radical scavenging (ii) benzene-ring methoxyl groups of phenols, especially A and B-ring methoxyl groups of the flavonol skeleton, decreased DPPH radical scavenging effects, as was observed for compounds **1** (EC_{50} = 26.2 μM), **5** (EC_{50} = 31.2 μM), **3** (EC_{50} = 48.3 μM), **6** (EC_{50} > 100 μM), and **7** (EC_{50} > 100 μM); and (iii) an A-ring glycosyl group of the flavone skeleton retained or slightly enhanced the antioxidant activity, as indicated by comparing the activities of compounds **1** and **5**.

Characterization of the crude FDL, FDS, ADL, and ADS extracts

Dry weights (DWs) of 30%, 50%, 70%, and 95% EtOH extracts of the freeze- and air-dried leaves and stems (FDL, FDS, ADL, and ADS, respectively) of PHT were measured. Regardless of drying type, the DWs of PHT leaf extracts were lower when extracted with higher percentages of EtOH, and the trend was similar for the extracts of stems (Table 2). The DWs of FDL extracts with same solvent were significantly greater than those of the ADL extracts (1.11- to 1.21-fold), but the DWs of FDS and ADS extracts with the same solvent did not have this tendency. In addition, the DWs of FDL extracts were greater than those of the FDS extracts (1.34- to 1.85-fold), and the DWs of the ADL extracts were greater than those of the ADS extracts (1.22- to 2.07-fold).

In regards to total phenolic and flavonoid content expressed as mg catechin equivalents (CE) per g extract, we found that the content of the extracts of the freeze- and air-dried leaves and stems (FDL, FDS, ADL, and ADS, respectively) of PHT increased with increasing concentrations of EtOH (Table 2). Regardless of drying type, the total phenolic and flavonoid content of PHT stem extracts were higher than those of PHT leaf extracts. In regards to drying types, the total phenolic and flavonoid content of the freeze-dried leaf and stem extracts (FDL and FDS, respectively) with same solvent were higher than those of air-dried leaf and stem extracts (ADL and ADS, respectively) with same solvent. In addition, the total phenolic content of FDS extracts were higher than those of the FDL extracts (1.15- to 2.48-fold), and the total flavonoid content of the FDS extracts were greater than those of the FDL extracts (1.02- to 2.43-fold). Among the extracts of the freeze- and air-dried leaves and stems, the 95% ethanol extracts of freeze-dried stems (95FDS) exhibited

Table 2 Dry weights and total phenolic and flavonoid content of 30%, 50%, 70%, and 95% ethanol extracts from freeze-dried and air-dried leaves and stems of PHT

Extracts	DW of extract (mg/g dried material)	Total phenolics (mg CE/g extract)	Total flavonoids (mg CE/g extract)
Leaf			
30FDL	324.6 ± 12.1[a]	44.4 ± 1.1[d]	20.7 ± 0.3[e]
50FDL	272.8 ± 4.7[c]	98.7 ± 0.0[c]	78.5 ± 1.3[c]
70FDL	250.4 ± 0.2[d]	148.0 ± 3.0[b]	116.6 ± 0.3[b]
95FDL	145.4 ± 3.2[f]	158.5 ± 0.2[a]	146.3 ± 0.3[a]
30ADL	286.8 ± 3.7[b]	20.1 ± 0.2[f]	6.6 ± 0.3[g]
50ADL	246.8 ± 5.4[d]	33.9 ± 1.3[e]	15.7 ± 0.0[f]
70ADL	207.0 ± 2.2[e]	45.7 ± 0.2[d]	17.0 ± 0.3[f]
95ADL	124.6 ± 4.0[g]	48.4 ± 0.2[d]	44.6 ± 0.5[d]
Stem			
30FDS	206.2 ± 1.0[a]	110.1 ± 0.2[d]	50.3 ± 0.8[e]
50FDS	203.9 ± 8.2[a]	140.4 ± 0.0[b]	83.5 ± 1.6[c]
70FDS	144.4 ± 1.2[c]	179.3 ± 0.0[a]	126.8 ± 0.0[b]
95FDS	78.8 ± 1.5[d]	182.1 ± 2.6[a]	149.3 ± 0.0[a]
30ADS	209.5 ± 2.7[a]	45.4 ± 1.5[f]	18.9 ± 0.3[g]
50ADS	202.5 ± 6.7[a]	56.9 ± 1.1[e]	20.7 ± 0.3[g]
70ADS	159.1 ± 4.2[b]	57.7 ± 2.2[e]	24.4 ± 0.3[f]
95ADS	60.2 ± 1.0[e]	130.4 ± 0.4[c]	55.9 ± 0.3[d]

Values indicate mean ± SD (n = 3). DW: dry weight; CE: catechin equivalents

FDL freeze-dried leaves, *FDS* freeze-dried stems, *ADL* air-dried leaves, *ADS* air-dried stems

[a–g] Means not sharing a common letter within a column indicate that extracts from the same parts of PHT are significantly different ($p < 0.05$) by one-way ANOVA

highest phenolic and flavonoid content, which were 1.40 and 2.67 times, respectively, greater than those of air-dried stems (ADS). Moreover, we found that the extracts of all four types of plant material contained more total phenolics than total flavonoids (1.08- to 3.05-fold), regardless of drying method and extraction solvent.

Next, the main components presented in the extracts from PHT were analysed and measured the content using a Shimadzu HPLC system. Compounds **1**–**8**, which were isolated from the extracts of PHT, were used as external standards for the HPLC analysis. The peaks (**1**–**8**) of the HPLC profiles were identified as compounds **1**–**8** (Figs. 3 and 4). For each extraction solvent, the peaks were generally higher in the 95% EtOH extracts of FDL, ADL, FDS, and ADS than in the 30%, 50%, and 70% EtOH extracts of them, at 10 mg/mL. In particular, we found that the extracts of freeze-dried material contained relatively higher concentrations of compounds **1** and **2**, whereas the extracts of air-dried material was primarily composed of compounds **7** and **8**. Because compounds **8** was not active in the inhibition of LDL-oxidation and DPPH radical scavenging, and the content of main components **1**, **2**, and **7** in each extract were measured (Table 3).

The calibration curves of compounds **1**, **2**, and **7** exhibited good linearity ($R^2 = 0.9986$ to 0.9997; Table 4), and the limits of detection and quantification ranged from 0.06 to 0.09 μg/mL and from 0.19 to 0.29 μg/mL, respectively. The amount of compound **1** was higher in the 50% and 70% EtOH extracts of leaf and stem regardless of drying type than in the 30% and 95% EtOH extracts of them, and was especially high in the 70% EtOH extract of FDS (12.67 mg/g extract).

Compound **2**, rosmarinic acid, is well known as a main constituent (91.8–154.6 mg/100 g of fresh material weight) of some herbs, including Mexican oregano (*Poliomintha longiflora*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) [33]. Rosmarinic acid exhibits antioxidant, anti-inflammatory, antiangiogenic, neuroprotective, antimicrobial, immunomodulatory, and anti-atherosclerotic activities, owing to its antioxidant and radical scavenging properties [34] and resulting in its use in the functional food industry. The amount of compound **2** in the extracts from freeze-dried material was clearly distinguished from the amounts in the extracts of air-dried material, and was significantly higher in the 95% extracts of FDS (116.05 mg/g extract) and FDL (67.87 mg/g extract) than in the other extracts of freeze-dried material. In another study, the rosmarinic acid content of EtOH extracts from freeze-dried lemon balm (*Melissa officialis* L.) leaves was greater than that that of the extracts of hot air-dried leaves [35, 36], which is in agreement with the results of the present study. In contrast, the rosmarinic acid content and antioxidant capacity of six Lamiaceous herbs were also investigated after drying the plant material with three different methods (air-dried for 3 weeks at 14 °C in a dark, well-ventilated room; vacuum oven-dried for 16 h at 70 °C and 600 mbar; or freeze-dried for 72 h at −54 °C and 0.064 mbar), and the authors found that both the rosmarinic acid content and antioxidant capacity were significantly higher in air-dried samples than vacuum oven-dried or freeze-dried samples throughout the 60-day storage period [37]. However, the 3-week, low-temperature air-drying method was different from the drying methods used in the present study, and the study did not investigate the rosmarinic acid content of *P. hadiensis* var. *tomentosus,* either.

In contrast to compounds **1** and **2**, the concentration of compound **7** was higher in ADS extracts than in the ADL extracts, as well as the FDS and FDL extracts, and was especially high in the 95% EtOH extract of ADS (23.86 mg/g extract). That would be linked to release flavonoids, mainly quercetin derivatives, through air-drying process [38].

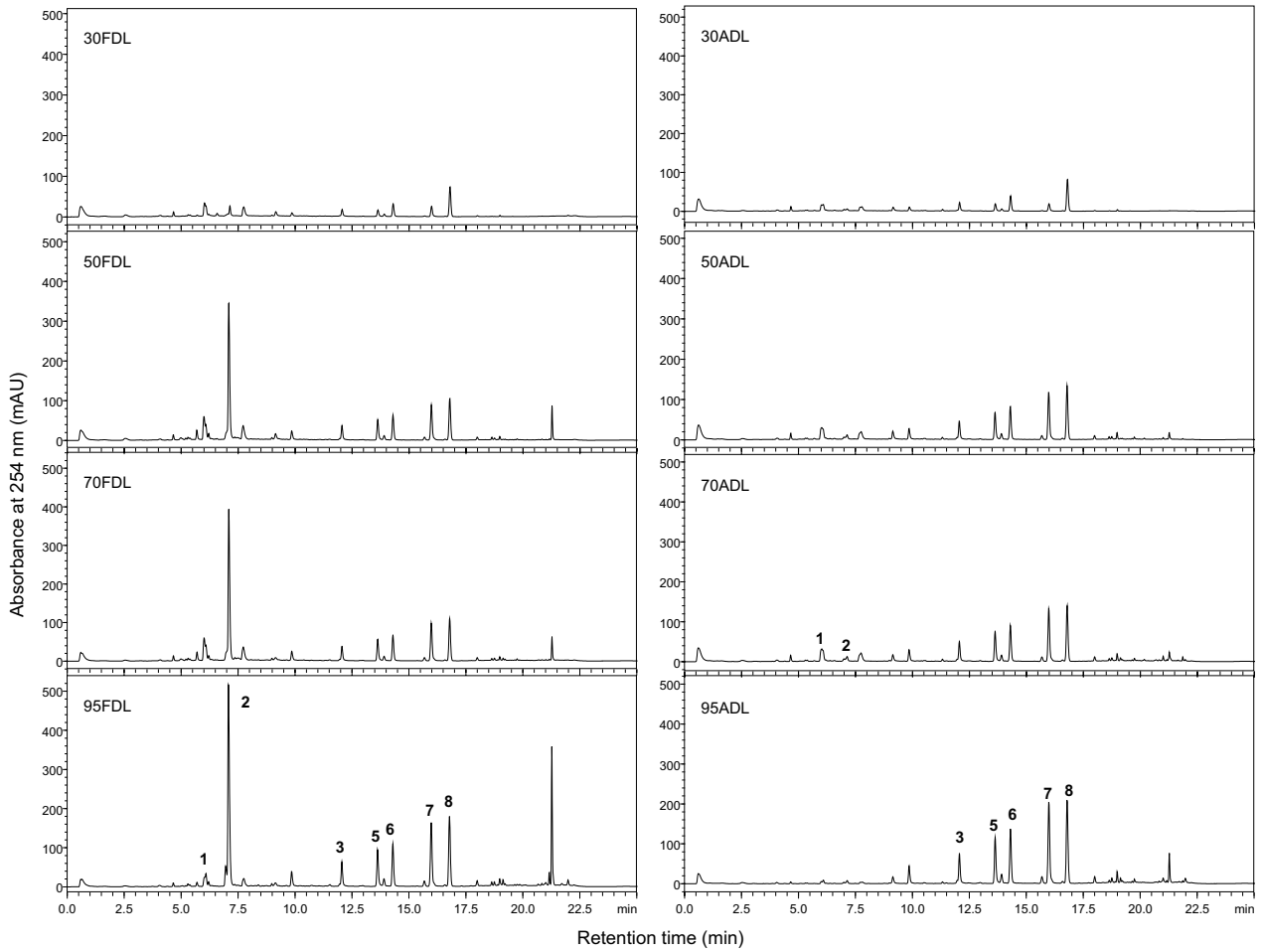

Fig. 3 HPLC chromatograms of 30%, 50%, 70%, and 95% ethanol extracts (10 mg/mL) from freeze-dried and air-dried leaves of PHT. *FDL* freeze-dried leaves, *ADL* air-dried leaves

Antioxidant activities of crude leaf and stem extracts from PHT

Throughout the present study, the drying method and extraction solvent were strongly correlated with the extraction of the three individual phenolic components, and higher content of compounds **1** and **2** may responsible for strong antioxidant activity of the extracts. Indeed, we found that the extracts with higher content of compounds **1** and **2** were more effective as LDL-antioxidants and DPPH radical scavengers than those with lower levels (Table 5).

Among the FDL extracts, the 95% EtOH extract exhibited the highest inhibition (66.4% at 5 μg/mL) of Cu^{2+}-induced oxidation of LDL, followed by the inhibition activity of the 70% (43.7% at 5 μg/mL), and 50% (33.0% at 5 μg/mL) EtOH extracts, respectively. Moreover, the 95% EtOH extract of FDS exhibited highest inhibition activity (91.9% at 5 μg/mL) of all the PHT stem extracts, followed by the inhibition activity of the 70% EtOH extract (82.6% at 5 μg/mL). In contrast, the extracts of ADL and ADS exhibited relatively weak inhibition activities (below 23.9% at 5 μg/mL). Thus, high content of compound **2** in the 70% and 95% EtOH extracts of FDL and FDS may responsible for strong LDL-antioxidant activity of the extracts.

In addition, the 95% and 70% EtOH extract of FDS also exhibited the very high DPPH radical scavenging activities (98.6% and 91.2% at 100 μg/mL, respectively).

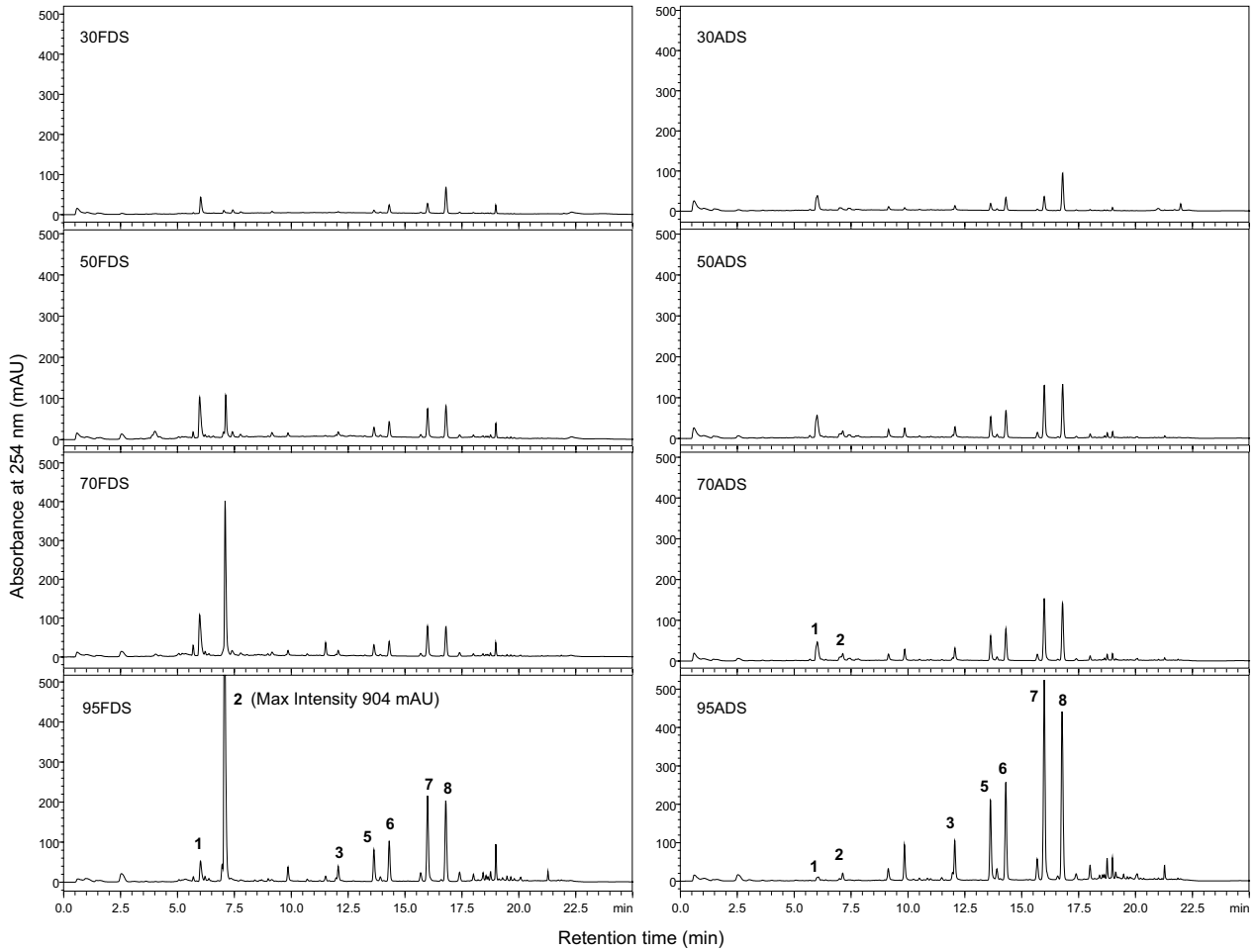

Fig. 4 HPLC chromatograms of 30%, 50%, 70%, and 95% ethanol extracts (10 mg/mL) from freeze-dried and air-dried stems of PHT. *FDS* freeze-dried stems, *ADS* air-dried stems

However, among the FDL extracts, the highest radical scavenging activity was exhibited by the 70% EtOH extract (86.9%), followed by the 50% (82.2%) and 95% (80.6%) EtOH extracts. The DPPH radical scavenging activities of the EtOH extracts of ADL and ADS was relatively lower, 21.2–27.6% inhibition and 32.0–41.9% at 100 μg/mL, respectively, than those of FDL and FDS. Thus, it seems that freeze-drying was more effective than air-drying in preserving the potent antioxidant activities of PHT, which may be related to the higher phenolic content of the extracts of freeze-dried material and the fact that compounds **7** and **8**, which were the major components of the extracts of air-dried material, exhibited relatively low antioxidant activity.

Considering the antioxidant properties demonstrated by both LDL-oxidation and DPPH radical scavenging assays, the 70–95% EtOH extract of freeze-dried tissues should be used for the development of a natural antioxidant from PHT.

The present study isolated compounds **1**–**8** from the EtOH extract of PHT leaves and stems, and characterized the content and antioxidant activity of 30–95% EtOH crude extracts of FDL, FDS, ADL, and ADS. Most importantly, LDL-antioxidant and DPPH radical scavenging activity of the flavonoids **3** and **5**–**7** were reported for the first time, and we suggest that the antioxidant activities of the EtOH extracts of FDL, FDS, ADL, and ADS resulted from the combination

Table 3 Content of compounds 1, 2, and 7 in the 30%, 50%, 70%, and 95% ethanol extracts from freeze-dried and air-dried leaves and stems of PHT

Extracts	Contents of compounds (mg/g extract)		
	1	**2**	**7**
Leaf			
30FDL	3.95 ± 0.02^{cd}	2.79 ± 0.01^{d}	1.38 ± 0.00^{h}
50FDL	7.25 ± 0.02^{b}	39.50 ± 0.00^{c}	3.55 ± 0.00^{f}
70FDL	8.36 ± 0.01^{a}	43.22 ± 0.00^{b}	3.76 ± 0.00^{e}
95FDL	1.61 ± 0.02^{f}	67.87 ± 0.00^{a}	7.54 ± 0.00^{b}
30ADL	2.12 ± 0.00^{e}	0.80 ± 0.00^{h}	1.79 ± 0.00^{g}
50ADL	3.75 ± 0.27^{d}	1.59 ± 0.00^{f}	5.91 ± 0.02^{d}
70ADL	4.18 ± 0.00^{c}	1.74 ± 0.00^{e}	6.42 ± 0.03^{c}
95ADL	0.58 ± 0.11^{g}	0.94 ± 0.01^{g}	10.85 ± 0.08^{a}
Stem			
30FDS	4.00 ± 0.26^{e}	1.05 ± 1.13^{e}	0.99 ± 0.07^{g}
50FDS	10.53 ± 0.92^{b}	13.11 ± 0.96^{c}	3.65 ± 0.33^{e}
70FDS	12.67 ± 0.57^{a}	48.99 ± 1.88^{b}	3.92 ± 0.19^{e}
95FDS	5.40 ± 0.10^{d}	116.05 ± 1.61^{a}	10.85 ± 0.09^{b}
30ADS	5.52 ± 0.03^{d}	1.78 ± 0.01^{de}	2.01 ± 0.00^{f}
50ADS	7.37 ± 0.02^{c}	3.22 ± 0.01^{d}	5.88 ± 0.03^{d}
70ADS	7.11 ± 0.00^{c}	3.51 ± 0.01^{d}	7.95 ± 0.03^{c}
95ADS	1.29 ± 0.01^{f}	2.32 ± 0.03^{de}	23.86 ± 0.24^{a}

Values indicate mean ± SD (n = 3)

FDL freeze-dried leaves, *FDS* freeze-dried stems, *ADL* air-dried leaves, *ADS* air-dried stems

[a–h] Means not sharing a common letter within a column indicate that extracts from the same parts of PHT are significantly different ($p < 0.05$) by one-way ANOVA

Table 5 Antioxidant activity of 30%, 50%, 70%, and 95% ethanol extracts from freeze-dried and air-dried leaves and stems of PHT

Extracts	LDL oxidation inhibition (%) at 5 μg/mL	DPPH radical scavenging (%) at 100 μg/mL
Leaf		
30FDL	NI	31.5 ± 0.1^{c}
50FDL	33.0 ± 0.0^{c}	82.2 ± 1.7^{b}
70FDL	43.7 ± 0.4^{b}	86.9 ± 1.6^{a}
95FDL	66.4 ± 1.4^{a}	80.6 ± 1.4^{b}
30ADL	NI	23.9 ± 1.1^{e}
50ADL	5.5 ± 0.0^{e}	27.6 ± 0.3^{d}
70ADL	4.7 ± 1.3^{e}	25.2 ± 0.7^{e}
95ADL	11.1 ± 1.7^{d}	21.2 ± 0.5^{f}
Stem		
30FDS	NI	31.4 ± 0.4^{e}
50FDS	5.8 ± 0.7^{e}	47.3 ± 1.2^{c}
70FDS	82.6 ± 1.4^{b}	91.2 ± 0.9^{b}
95FDS	91.9 ± 0.3^{a}	98.6 ± 0.3^{a}
30ADS	4.3 ± 0.9^{d}	32.0 ± 0.0^{f}
50ADS	16.5 ± 3.5^{c}	41.9 ± 1.6^{e}
70ADS	15.7 ± 0.9^{d}	39.0 ± 2.2^{e}
95ADS	23.9 ± 1.6^{b}	39.0 ± 1.6^{e}

Values indicate mean ± SD (n = 3). NI: not inhibited

FDL freeze-dried leaves, *FDS* freeze-dried stems, *ADL* air-dried leaves, *ADS* air-dried stems

[a–f] Means not sharing a common letter within a column indicate that extracts from the same parts of PHT are significantly different ($p < 0.05$) by one-way ANOVA

of compounds **1**–**3** and **5**–**7**. In the present study, the 95% EtOH extract of FDS exhibited the highest total phenolic and flavonoid content, as well as the highest antioxidant activity, as indicated by both TBARS and DPPH radical scavenging assays. However, when considering extraction yield, total phenolic and flavonoid content, and antioxidant activity together, the 70% EtOH was more suitable and effective for extraction from freeze-dried material. Thus, our findings support the idea that abundant flavonoids and phenolic acids could have beneficial antioxidant effects in PHT, such beneficial effects would mainly be provided by compound **2**, rosmarinic acid, which was the most potent LDL-antioxidant and DPPH radical scavenger among the eight compounds. These findings are important for the development of PHT as an effective natural antioxidant material, which is contained rich phenolic and flavonoid compounds.

Table 4 Quantitative analysis of compounds 1, 2, and 7 in PHT using HPLC–DAD

Compounds	Linear regression data				LOD (μg/mL)	LOQ (μg/mL)
	Calibration curve	R^2	SD	RSD%		
1	y = 5814.468x + 6319.507	0.9997	108.74	1.84	0.06	0.19
2	y = 4100.056x + 7260.455	0.9993	84.70	2.02	0.07	0.21
7	y = 10,254.18x + 22,178.86	0.9986	293.48	2.76	0.09	0.29

Values indicate mean ± SD from two independent experiments performed in triplicate. The limit of detection (LOD) and quantification (LOQ) were determined according to the International Conference of Harmonization (ICH)-Q2 guidelines (ICH. 2005). LOD = 3.3 δ/S and LOQ = 10 δ/S, where δ = SD of the response and S = slope of the calibration curve

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13765-019-0464-y.

Additional file 1: Figure S1. HPLC chromatograms of 95% EtOH extract (A) and *n*-hexane (B), $CHCl_3$ (C), and 10% aqueous MeOH (D) layers from the aerial parts of *Plectranthus hadiensis* var. *tomentosus*. Figure S2–S4: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **1.** Figure S5–S7: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **2.** Figure S8–S10: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **3.** Figure S11–S13: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **4.** Figure S14–S16: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **5.** Figure S17–S19: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **6.** Figure S20–S22: ESI–MS, ^{1}H NMR, and ^{13}C NMR spectra of compound **7.** Figure S23–S26: ESI–MS, ^{1}H NMR, ^{13}C NMR, and NOE spectra of compound **8.**

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Authors' contributions
TSJ, HSJ, and HL participated in research design and writing the manuscript; HSJ, HL, EJM, and UHK conducted the experiments; HSJ, HL, YHK, HYP, and TSJ performed data analysis. All authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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