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Chemical Components and Tyrosinase Inhibitors from the Twigs of *Artocarpus heterophyllus*

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An HPLC method was developed and validated to compare the chemical profiles and tyrosinase inhibitors in the woods, twigs, roots, and leaves of *Artocarpus heterophyllus*. Five active tyrosinase inhibitors including dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin were used as marker compounds in this HPLC method. It was discovered that the chemical profiles of *A. heterophyllus* twigs and woods are quite different. Systematic chromatographic methods were further applied to purify the chemicals in the twigs of *A. heterophyllus*. Four new phenolic compounds, including one isoprenylated 2-arylbenzofuran derivative, artoheterophyllin A (**1**), and three isoprenylated flavonoids, artoheterophyllin B (**2**), artoheterophyllin C (**3**), and artoheterophyllin D (**4**), together with 16 known compounds, were isolated from the ethanol extract of the twigs of *A. heterophyllus*. The structures of compounds **1**−**4** were elucidated by spectroscopic analysis. However, the four new compounds did not show significant inhibitory activities against mushroom tyrosinase compared to kojic acid. It was found that similar compounds, such as norartocarpetin and artocarpesin in the twigs and woods of *A. heterophyllus*, contributed to their tyrosinase inhibitory activity.

KEYWORDS: *Artocarpus heterophyllus*; twigs; phenolic compounds; tyrosinase inhibitors; chemical constituents

INTRODUCTION

Tyrosinase (EC 1.14.18.1), or polyphenol oxidase, occurs widely in plants and animals. In humans, it is involved in the formation of melanin pigments (*1*) and catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, which have been identified as the initial steps in melanin formation (*2*−*4*). In the food industry, postharvest browning is an important cause of economic loss. Several factors, such as enzymatic oxidation of phenols catalyzed by tyrosinase, the Maillard reaction, and ascorbic acid oxidation, can cause browning when food is processed and/or stored (*5*). Enzymatic browning in food could lead to significant changes in the appearance, flavor, and nutritional quality (*6*). Such undesirable deterioration in quality attributes could be prevented by blanching or adding chemical additives at certain stages of food processing. However, blanching can cause unwanted softening of the tissues (*7*). Chemical additives, such as sulfites, ascorbic acid, and citric acid, were used as antibrowning agents in the past, but they were not used extensively in food because of their adverse effects or low inhibitory activities (*8*). Sulfites, in particular, were banned in 1986 due to its potential health hazards to sensitive individuals. Presently, the public is becoming ever more concerned about the potential health risk of synthetic food additives. Therefore, there is a great demand from the food and cosmetic industry for safe and effective tyrosinase inhibitors of natural origins. Recently, our group has identified novel natural tyrosinase inhibitors from plants of the genus *Morus*. Several potent tyrosinase inhibitors were isolated from the twigs of *Morus alba* (*9*) and the wood of *Artocarpus heterophyllus* (*10*). In the wood of *A. heterophyllus*, five compounds, including dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin, were found to be the major tyrosinase inhibitors. In addition, we discovered significant antibrowning effects of *M. alba* twig and *A. heterophyllus* wood extracts on fresh-cut apple slices when they were applied together with vitamin C (*9*, *10*). However, to the best of our knowledge, the tyrosinase inhibitors and the chemical components in the twig part of *A. heterophyllus* have not been well characterized. In preliminary tests, we found that the tyrosinase inhibitory activity of the wood extract of *A. heterophyllus* was the highest, followed by the root, twig, and leaf extracts. It is of great interest to investigate whether the roots, twigs, leaves, and woods contain similar active components or whether other novel tyrosinase inhibitors are present. To this end, we developed an HPLC method with the five tyrosinase inhibitors (dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin) identified in the woods of *A. heterophyllus* as marker compounds to compare the chemical profiles of the wood,

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twigs, roots, and leaves. To further identify the chemical components in the twigs, systematic chromatographic and spectral approaches were applied to isolate and characterize the compounds in the twigs of *A. heterophyllus*. Here we report the HPLC method for the analysis of tyrosinase inhibitors in different parts of *A. heterophyllus* and characterization of four new natural products isolated from the twigs of *A. heterophyllus*.

MATERIALS AND METHODS

Chemicals and General Procedures. Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). MCI gel CHP 20P (75−150 μm) was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan). LiChroprep RP-18 (40−63 μm) and TLC plates (25 DC-platten Kieselgel 60 F_{254}) were from Merck (Darmstadt, Germany). Silica gel (200−300 mesh) for column chromatography was from Anhui Liangchen Slicon Material Co., Ltd. (Anhui, China). Amberlite XAD16 was purchased from Sigma-Aldrich (St. Louis, MO). Mushroom tyrosinase (5370 units/mg), L-tyrosine, and boric acid were purchased from Sigma-Aldrich. HPLC grade solvents were from BDH (Poole, U.K.). The semipreparative HPLC system was carried on a Waters 600 system equipped with a 2487 dual-wavelength detector, Masslynx V4.0 software, and a Phenomenex Luna C_{18} (2) column (250 × 21.2 mm, 5 μm). Analytical HPLC was performed on a Shimadzu HPLC system with a separation module (LC-20AT), an autosampler (SIL-20A), a degasser (DGU-20A3), a photodiode array detector (SPD-M20A), and LC-solution software. ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, and HMBC spectra were obtained on a Bruker 400 or 500 DRX NMR spectrometer. Molecular weights of all compounds were analyzed on an LC-MS instrument equipped with an electrospray ionization source interfaced to a Q-trap mass spectrometer (Applied Biosystems). Tyrosinase inhibitory activity was measured on a UV-1206 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Plant materials were ground with a minigrinder (model DF-15, Shenzhen Laitong Co., Shenzhen, China). Powdered plant materials were extracted with a traditional Chinese medicine extractor (model YFX20T, Donghuayuan Medical Co., Beijing, China).

Plant Material. Fresh twigs, leaves, and roots of *A. heterophyllus* were collected at Wenchang County, Hainan Province, China, in October 2007. Fresh woods of *A. heterophyllus* were collected from the same place in October 2006. Voucher specimens were deposited at the School of Biological Sciences, The University of Hong Kong.

HPLC Analysis of *A. heterophyllus* Samples. HPLC analyses were carried out on an Alltima C_{18} column (250 × 4.6 mm, 5 μm, Delta Technical Products Co., Des Plaines, IL). The mobile phase was composed of 0.2% formic acid in water (solvent A) and acetonitrile (solvent B). The elution program was as follows: initial, 20% B; 0−20 min, 20−40% B; 20−30 min, 40−60% B; 30−40 min, 60−80% B; 40−50 min, 80−100% B. The postrunning time was 10 min. Flow rate was set at 1.0 mL/min. The UV−vis detector was set at 285 nm. The column was maintained at room temperature. Injection volume was 10 μL. Triplicate analyses were performed for each sample.

Calibration Curves. Methanol stock solutions containing the five active compounds (dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin) were prepared and diluted to final concentrations of 1.25, 2.5, 5, 10, 25, 50, 100, and 200 μg/mL. A calibration curve was constructed for each of the compounds by plotting peak areas versus compound concentrations.

Reproducibility of the HPLC Method. Five injections of the same standard mixture and extract solution were made, and coefficients of variance (CV) of retention times and peak areas of the above five compounds were analyzed to determine the reproducibility of the HPLC method.

Recovery of the HPLC Method. Extraction recovery was determined for the five compounds. Samples of wood extract were spiked with the five compounds at 1.2×, 1×, and 0.8× amounts. Duplicate analyses were performed, and percentage recoveries were calculated.

Preparation of *A. heterophyllus* Extracts. Fifteen hundred milligrams each of *A. heterophyllus* wood, twig, root, and leaf powders was accurately weighed into a conical flask. Forty-five milliliters of methanol was added to each flask, and the suspensions were sonicated for 60 min. After cooling, methanol was added to fill to the graduation mark. The extracts were filtered before HPLC analysis.

Isolation and Purification of Compounds from the Twigs of *A. heterophyllus*. The twigs of *A. heterophyllus* (9.5 kg) were ground with a minigrinder and packed into a paper extraction bag. Extraction was performed with 95% ethanol (3 × 15 L) at room temperature using a traditional Chinese medicine extractor. The ethanol extracts were combined and concentrated on a rotary evaporator at 45 °C under vacuum. The dried extract was then suspended in water and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate-soluble part (163.0 g) showed the strongest tyrosinase inhibitory activity (IC_{50} = 9.4 μg/mL) and was thus subjected to repeated column chromatography [silica gel (200−300 mesh) eluted with *n*-hexane/ethyl acetate (30:1, 10:1, 5:1, 2:1, 1:1, v/v), ethyl acetate, ethyl acetate/methanol (1:1), and finally methanol], which led to eight fractions (1−8). Fraction 3 (3.4 g) was separated with an Amberlite XAD16 column, eluted with 50, 70, and 95% ethanol, to obtain three subfractions (a1−a3). Fractions a2 and a3 were further separated by MCI gel column chromatography (MeOH/H_2O, 80:20, 90:10, 100:0) to afford 20 fractions (1′−20′). Fractions 8′−10′, 11′−12′, 13′−16′, and 19′ were separated respectively with a Sephadex LH-20 column (eluted with MeOH/H_2O, 1:1, v/v) and further purified by preparative HPLC. Purification of fractions 8′−10′ gave compounds **17** (8.0 mg) and **18** (3.0 mg), that of fractions 11′−12′ gave compounds **14** (5.6 mg), **3** (2.0 mg), and **20** (2.0 mg), that of fractions 13′−16′ gave compound **4** (3.0 mg), and that of fraction 19′ gave compound **19** (16.6 mg). Fractions 17′−18′ were purified by Sephadex LH-20 column chromatography (eluted by MeOH/H_2O, 1:1) to offer compound **16** (14.0 mg). Fraction 4 (2.8 g) was separated with an Amberlite XAD16 column (eluted with 30, 50, 70, and 95% ethanol) to offer four subfractions (b1−b4). Fraction b3 was purified with a Sephadex LH-20 column (eluted with MeOH/H_2O, 1:1, v/v) and further purified by preparative HPLC to get compounds **12** (34.8 mg) and **13** (33.0 mg). Fraction b4 was fractionated by Sephadex LH-20 column chromatography (eluted with MeOH/H_2O, 1:1, v/v), which led to 14 fractions (1″−14″). Similar fractions were combined. Fractions 10″−11″ were separated by MCI gel column chromatography (MeOH/H_2O, 70:30, 80:20, 90:10, 100:0) to give 19 fractions (1‴−19‴). Fractions 3‴−4‴ was purified by preparative HPLC to offer compound **1**. Preparative HPLC of fraction 6‴ (MeOH/H_2O, 80:20) gave compound **15** (4.0 mg). Fractions 15‴−18‴ were purified by Sephadex LH-20 column chromatography (eluted by MeOH/H_2O, 1:1, v/v) and then by preparative HPLC to offer compound **2** (10.0 mg). Fraction 5 (1.5 g) was separated with an Amberlite XAD16 column (eluted with 10, 30, 50, and 70% ethanol) into four subfractions (c1−c4). Fraction c4 was fractionated by Sephadex LH-20 chromatography (eluted by MeOH/H_2O, 1:1) and further purified by preparative HPLC to obtain compound **11** (39.2 mg). Fraction 6 (1.2 g) was fractionated by Amberlite XAD16 column chromatography (eluted with 10, 30, 50, and 70% ethanol) into three subfractions (d1−d3). Fraction d3 was separated with a Sephadex LH-20 column (eluted by MeOH/H_2O, 1:1, v/v) and further purified by preparative HPLC to offer compounds **8** (23.3 mg) and **10** (2.0 mg). Fraction 7 (1.8 g) was separated with an Amberlite XAD16 column (eluted with 10, 30, 50, and 70% ethanol) into four subfractions (e1−e4). Fraction e1 was purified successively by Sephadex LH-20 (eluted by MeOH/H_2O, 1:1, v/v) and preparative HPLC to get compounds **6** (24.2 mg) and **9** (2.0 mg). Fraction 7 (0.3 g) was separated by Amberlite XAD16 column chromatography (eluted with 10, 30, 50, and 70% ethanol) into four subfractions (f1−f4). Fraction f1 was purified successively by Sephadex LH-20 (eluted with MeOH/H_2O, 1:1) and preparative HPLC to get compound **7** (3.0 mg). Sephadex LH-20 (eluted by MeOH/H_2O, 1:1) and preparative HPLC of fraction f2 led to compound **5** (11.7 mg).

Artoheterophyllin A (***1***)*:* yellow powder; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.71 (1H, s, H-7), 6.57 (1H, d, *J* = 2.8 Hz, H-6′), 6.29 (1H, s, H-3), 6.28 (1H, d, *J* = 2.4 Hz, H-4′), 5.23 (1H, m, H-2‴), 5.09 (1H, m, H-2″), 3.74 (3H, s, OCH_3), 3.46 (2H, d, *J* = 6.8 Hz, H-1″), 3.37 (2H, d, *J* = 6.8 Hz, H-1‴), 1.75 (3H, s, CH_3), 1.58 (6H, s, 2 × CH_3), 1.56 (3 H, s, CH_3); ^{13}C NMR (CD_3OD, 100 MHz) δ 156.5 (C, C-3′), 155.4 (C, C-7a, 5), 154.9 (C, C-2), 153.8 (C, C-5′), 148.4 (C, C-6), 131.8 (C, C-1′), 130.2 (C, C-3″), 130.0 (C, C-3‴), 124.3 (CH, C-2″), 123.0 (CH, C-2‴), 117.9 (C, C-2′), 111.8 (C, C-3a), 106.4 (CH, C-6′), 104.7 (C, C-4), 102.3 (CH, C-4′), 101.6 (CH, C-7), 93.9 (CH, C-3), 55.6 (CH_3, OCH_3), 25.2 (CH_2, C-1″), 24.6 (CH_3, C-4″,

Table 1. HPLC Calibration Curve Data for the Five Active Tyrosinase Inhibitors

compd	retention time (min)	linear range (μg/mL)	calibration eq	correlation coefficient (R)	detection limit (μg/mL)
dihydromorin	11.493	1.25−200	$Y = 24787.4x + 13149.1$	0.9998	0.060
steppogenin	18.913	1.25−200	$Y = 32099.7x + 19980.8$	0.9998	0.040
norartocarpetin	19.463	1.25−200	$Y = 17125.4x + 4173.0$	0.9998	0.060
artocarpanone	27.482	1.40−224	$Y = 31504.2x + 20035.0$	0.9997	0.044
artocarpesin	32.494	1.38−220	$Y = 17324.4x + 7512.7$	0.9998	0.066

Table 2. Reproducibility Data of the Five Active Compounds for the HPLC Method ($n = 5$)

	retention time (min)		retention time CV (%)		peak area CV (%)	
compd	pure compd	extract	pure compd	extract	pure compd	extract
dihydromorin	11.493	11.502	0.372	0.482	0.753	0.949
steppogenin	18.913	18.927	0.260	0.211	0.749	2.048
norartocarpetin	19.463	19.471	0.234	0.197	0.959	1.034
artocarpanone	27.482	27.486	0.084	0.068	0.740	0.976
artocarpesin	32.494	32.491	0.055	0.037	0.721	1.129

C-4‴), 21.9 (CH_2, C-1‴), 16.7 (CH_3, C-5″), 16.6 (CH_3, C-5‴); ESI-MS m/z 409.2 $[M + H]^+$.

Artoheterophyllin B (**2**)*:* yellow powder; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.17 (1H, s, H-3′), 6.34 (1H, s, H-6′), 6.09 (1H, d, J = 9.3 Hz, H-1″), 5.43 (1H, d, J = 9.3 Hz, H-2″), 5.24 (1H, t, J = 6.6 Hz, H-2⁗), 5.18 (1H, t, J = 6.9 Hz, H-2‴), 3.56 (2H, m, H-1⁗), 3.34 (2H, d, J = 6.9 Hz, H-1‴), 1.93 (3H, s, H-4″), 1.85 (3H, s, H-4⁗), 1.76 (3H, s, H-4‴), 1.69, 1.68 (each 3H, H-5″,5⁗), 1.64 (3H, s, H-5‴); ^{13}C NMR (CD_3OD, 100 MHz) δ 157.7 (C, C-2), 110.6 (C, C-3), 180.2 (C, C═O, C-4), 158.3 (C, C-5), 113.3 (C, C-6), 160.4 (C, C-7), 108.4 (C, C-8), 153.9 (C, C-9), 106.1 (C, C-10), 108.4 (C, C-1′), 152.8 (C, C-2′), 105.7 (CH, C-3′), 153.3 (C, C-4′), 144.2 (C, C-5′), 110.5 (CH, C-6′), 70.6 (CH, C-1″), 122.9 (CH, C-2″), 139.8 (C, C-3″), 19.0, 26.3 (CH_3, C-4″,5″), 22.8 (CH_2, C-1‴), 123.6 (CH, C-2‴), 133.1 (C, C-3‴), 18.3, 26.2 (CH_3, C-4‴,5‴), 23.1 (CH_2, C-1⁗), 123.9 (CH, C-2⁗), 133.4 (C, C-3⁗), 18.6, 26.3 (CH_3, C-4⁗,5⁗); ESI-MS m/z 503.4 $[M-H]^-$.

Artoheterophyllin C (**3**)*:* yellow powder;^{1}H NMR (CD_3OD, 400 MHz) δ 6.24 (1H, s, H-3′), 5.28, 5.20 (each 1H, m, H-2‴,2⁗), 3.75 (1H, dd, J = 15.3, 7.7 Hz, H-1⁗), 3.47 (1H, dd, J = 14.8, 7.4 Hz, H-1⁗), 3.37 (1H, dd, J = 12.5, 7.0 Hz, H-2″), 3.36 (2H, d, J = 7.0 Hz, H-1‴), 3.18 (1H, dd, J = 15.2, 7.2 Hz, H-1″), 2.36 (1H, t, J = 15.1 Hz, H-1″), 1.79, 1.67 (each 3H, s, H-4‴,5‴), 1.79, 1.66 (each 3H, s, H-4⁗, 5⁗), 1.65, 1.32 (each 3H, s, H-4″, 5″); ^{13}C NMR (CD_3OD, 100 MHz) δ 162.7 (C, C-2), 112.6 (C, C-3), 182.6 (C, C-4, C═O), 157.9 (C, C-5), 113.2 (C, C-6), 160.4 (C, C-7), 108.4 (C, C-8), 153.9 (C, C-9), 105.6 (C, C-10), 105.5 (C, C-1′), 152.7 (C, C-2′), 105.4 (CH, C-3′), 138.2 (C, C-4′), 147.8 (C, C-5′), 134.2 (C, C-6′), 21.3 (CH_2, C-1″), 48.4 (CH, C-2″), 94.5 (C, C-3″), 28.7, 23.2 (CH_3, C-4″,5″), 22.8 (CH_2, C-1‴), 123.7 (CH, C-2‴), 132.9 (C, C-3‴), 18.3, 26.3 (CH_3, C-4‴,5‴), 22.9 (CH_2, C-1⁗), 124.1 (CH, C-2⁗), 133.4 (C, C-3⁗), 18.4, 26.3 (CH_3, C-4⁗,5⁗); ESI-MS m/z 503.3 $[M-H]^-$.

Artoheterophyllin D (**4**)*:* yellow powder; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.07 (1H, d, J = 8.3 Hz, H-6′), 6.70 (1H, d, J = 10.0 Hz, H-2‴), 6.42 (1H, d, J = 2.2 Hz, H-3′), 6.39 (1H, dd, J = 8.3, 2.2 Hz, H-5′), 5.68 (1H, d, J = 10.0 Hz, H-1‴), 5.13 (1H, t, J = 7.2 Hz, H-2⁗), 5.12 (1H, m, H-2″), 3.30 (2H, overlapped, H-1⁗), 3.10 (2H, d, J = 6.9 Hz, H-1″), 1.60 (6H, s, H-4⁗,5⁗), 1.58 (3H, s, H-4″), 1.40 (6H, s, H-4‴,5‴), 1.28 (3H, s, H-5″); ^{13}C NMR (CD_3OD, 125 MHz) δ 164.0 (C, C-2), 122.0 (C, C-3), 184.5 (C, C-4), 155.5 (C, C-5), 106.2 (C, C-6), 157.9 (C, C-7), 108.8 (C, C-8), 156.7 (C, C-9), 106.2 (C, C-10), 113.7 (C, C-1′), 162.2 (C, C-2′), 104.1 (CH, C-3′), 158.3 (C, C-4′), 108.2 (CH, C-5′), 132.7 (CH, C-6′), 25.2 (CH_2, C-1″), 123.2 (CH, C-2″), 133.1 (C, C-3″), 18.0, 26.1 (CH_3, C-4″,5″), 22.5 (CH_2, C-1⁗), 123.6 (CH, C-2⁗), 132.5 (C, C-3⁗), 18.2, 26.2 (CH_3, C-4⁗,5⁗), 117.0 (CH, C-1‴), 129.4 (CH, C-2‴), 79.1 (C, C-3‴), 28.8 (CH_3, C-4‴,5‴); ESI-MS m/z 487.3 $[M-H]^-$.

Mushroom Tyrosinase Inhibitory Assay. Mushroom tyrosinase inhibitory activity of plant extracts and isolated compounds was determined by spectrophotometric method using L-tyrosine as a substrate. The method was adopted from Vanni et al.'s study (*11*) with slight modifications. First, the extracts and compounds to be tested were dissolved in DMSO at 1.0 mg/mL. The solutions were diluted to different concentrations. Second, 30 μL of each sample solution or DMSO (control) was diluted with 970 μL of 0.05 mM sodium phosphate buffer (pH 6.8) in test tubes, followed by the addition of 1 mL of L-tyrosine solution and finally 1.0 mL of mushroom tyrosinase solution (200 units/mL). Third, the test mixtures (3.0 mL) were vortex-mixed, and absorbance was taken at 490 nm. Absorbance at the same wavelength was taken after incubation at 37 °C for 20 min. IC_{50}, the concentration of plant extract or compound at which half the original tyrosinase activity is inhibited, was determined. Percent inhibition of tyrosinase activity was calculated as

$$\%\text{inhibition} = [(A_2 - A_1) - (B_2 - B_1)]/(A_2 - A_1) \times 100$$

where A_1 is the absorbance at 490 nm without a test compound at 0 min, A_2 is the absorbance at 490 nm without a test compound at 20 min, B_1 is the absorbance at 490 nm with a test compound at 0 min, and B_2 is the absorbance at 490 nm with a test compound at 20 min.

Table 3. Recovery Data of the Five Active Compounds in the Twig Samples

compd	content (μg/g in wood extract)	added (μg)	found (μg)	recovery (%)	mean of recovery (%)	RSD (%)
dihydromorin	755 ± 11	906.0	908.62	100.29	99.85	0.944
		906.0	903.22	99.69		
		755.0	755.45	100.06		
		755.0	742.32	98.32		
		604.0	611.06	101.17		
		604.0	601.24	99.54		
steppogenin	127 ± 4	153.6	152.36	99.19	99.03	0.469
		153.6	151.80	98.83		
		128.0	127.68	99.75		
		128.0	126.83	99.09		
		102.4	101.36	98.98		
		102.4	100.69	98.33		
norartocarpetin	154 ± 4	187.2	184.39	98.50	99.36	0.846
		187.2	183.80	98.18		
		156.0	155.77	99.85		
		156.0	155.26	99.53		
		124.8	125.22	100.34		
		124.8	124.51	99.77		
artocarpanone	127 ± 2	152.4	150.08	98.48	99.84	1.081
		152.4	150.69	98.88		
		127.0	126.84	99.87		
		127.0	128.21	100.95		
		101.6	102.80	101.18		
		101.6	101.27	99.68		
artocarpesin	177 ± 3	213.6	213.08	99.76	99.70	1.130
		213.6	217.55	101.85		
		178.0	175.70	98.71		
		178.0	177.13	99.51		
		142.4	140.81	98.88		
		142.4	141.70	99.51		

RESULTS AND DISCUSSION

In this study, an HPLC method was developed to accurately analyze several known tyrosinase inhibitors that might be present

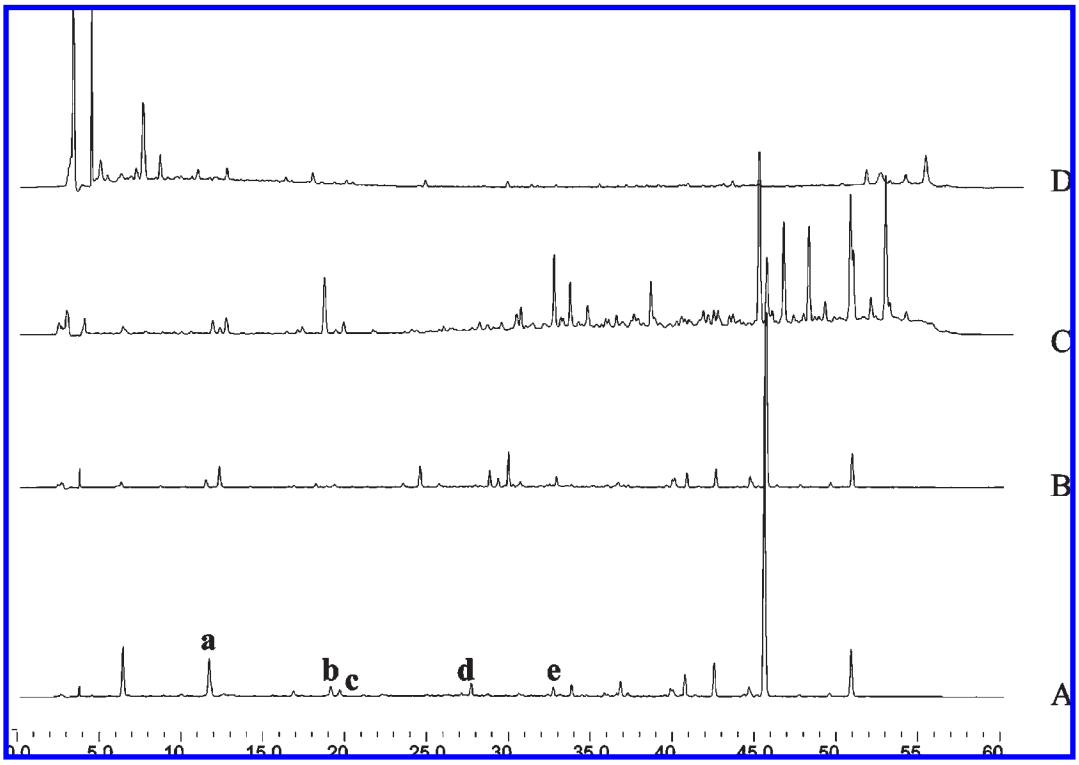

Figure 1. HPLC chromatogram of the extract from the woods (**A**), roots (**B**), twigs (**C**), and leaves (**D**) of *A. heterophyllus* [dihydromorin (a), steppogenin (b), norartocarpetin (c), artocarpanone (d), artocarpesin (e)], respectively.

in the wood, twigs, leaves, and roots of *A. heterophyllus*. The detection wavelength of 285 nm was selected on the basis of the UV spectra of the marker compounds dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin. Separation of these five compounds and other compounds was achieved with a gradient solvent system. On the basis of the validation data (**Table 1**−**3**) on the calibration curves, reproducibility, and recovery, this method is accurate and sensitive for the analysis of the five active components. The method was thus applied to analyze the above five tyrosinase inhibitors in the wood, twig, root, and leaf extracts of *A. heterophyllus*. The wood was found to contain the highest amount of these five compounds. The contents of dihydromorin, steppogenin, norartocarpetin, artocarpanone, and artocarpesin were determined to be 755, 127, 154, 127, and 177 μg/g of dry wood powder, respectively. As shown in **Figure 1**, constituents of roots were similar to those of the woods, and peaks corresponding to dihydromorin, steppogenin, norartocarpetin, and artocarpesin could easily be identified from their UV absorption spectra. Some of these five compounds were also found in the twigs, but their contents were lower. On the other hand, the constituents of the leaves were quite different from those of the woods, twigs, and roots, and these five major tyrosinase inhibitors were absent.

Moreover, HPLC analysis of the twig extract revealed a chemical profile that is quite different from that of the root and wood extracts. A number of unidentified peaks were observed in the spectrum of the twig extract. To identify these unknown components, a 95% ethanol extract of the twig was separated by column chromatography, and 20 compounds were isolated, including 4 new compounds and 16 known compounds. Their structures were characterized by 1D and 2D NMR spectroscopy and by comparison of the spectral data with the literature (the structures are shown in **Figure 2**).

The molecular formula of compound **1** was deduced as $C_{25}H_{28}O_5$ on the basis of ESI-MS (*m*/*z* 409.2 $[M+H]^+$) and ^1H NMR and ^{13}C NMR data. The ^1H NMR and ^{13}C NMR spectrum of **1** suggested that it might be an isoprenylated 2-arylbenzofuran, similar to the structure of artoindonesianins X and Y from *A. fretessi* (*12*, *13*). The signals in the ^1H NMR spectrum of **1** were assignable to a tetrasubstituted benzofuran moiety (δ 6.31, 6.71), a tetrasubstituted phenyl (δ 6.57, 6.28), two isoprenyl groups [δ 5.23, 5.09 ($2\times$CH=), 3.46, 3.37 (CH_2), 1.72, 1.58, 1.56 ($4\times CH_3$)], and one methoxy group at δ 3.74. Further evidence to support the structure of **1** was obtained from DEPT, HSQC, and HMBC spectral data. The HMBC spectrum revealed long-range correlations between the methylene signal at δ 3.46 and carbon signals at δ 131.8 (C-1′), 117.9 (C-2′), and 156.5 (C-3′), confirming the location of one isoprenyl group at C-2′. In the HMBC spectrum (**Figure 3**), the signal assigned to another methylene group at δ 3.37 showed strong correlations with carbon signals at δ 104.7 (C-4), 111.8 (C-3a), 155.4 (C-5), confirming the location of another isoprenyl group at C-4. Moreover, the methoxy group at δ 3.74 also showed strong correlation with the carbon signal at δ 155.4 (C-5), so the methoxy group was assigned to the location at C-5. In addition, the proton at δ 6.71 showed strong correlations with the carbon signals at δ 111.8 (C-3a) and 155.4 (C-5) and weak correlations with the carbon signal at δ 148.4 (C-6), suggesting the presence of one hydroxyl group at C-7. From the above data, compound **1** was elucidated as 5-(6-hydroxy-5-methoxy-4-(3-methylbut-2-enyl)-benzofuran-2-yl)-4-(3-methylbut-2-enyl) benzene-1,3-diol, named artoheterophyllin A.

The molecular formula of compound **2** was deduced as $C_{30}H_{32}O_7$ from the ESI-MS (*m*/*z* 503.4 $[M-H]^-$) and NMR data. When compared with literature data (*14*), the ^1H and ^{13}C NMR spectra of **2** were similar to those of artelastin, with the possible presence of an extra hydroxyl group on C-5′ of the B ring. In the ^1H NMR spectrum of **2**, ring B exhibited 1,2,4,5-tetrasubstitution with only two proton signals at δ 7.17 (H-3′) and δ 6.34 (H-6′). The characteristic signals of the isoprenyl side chain

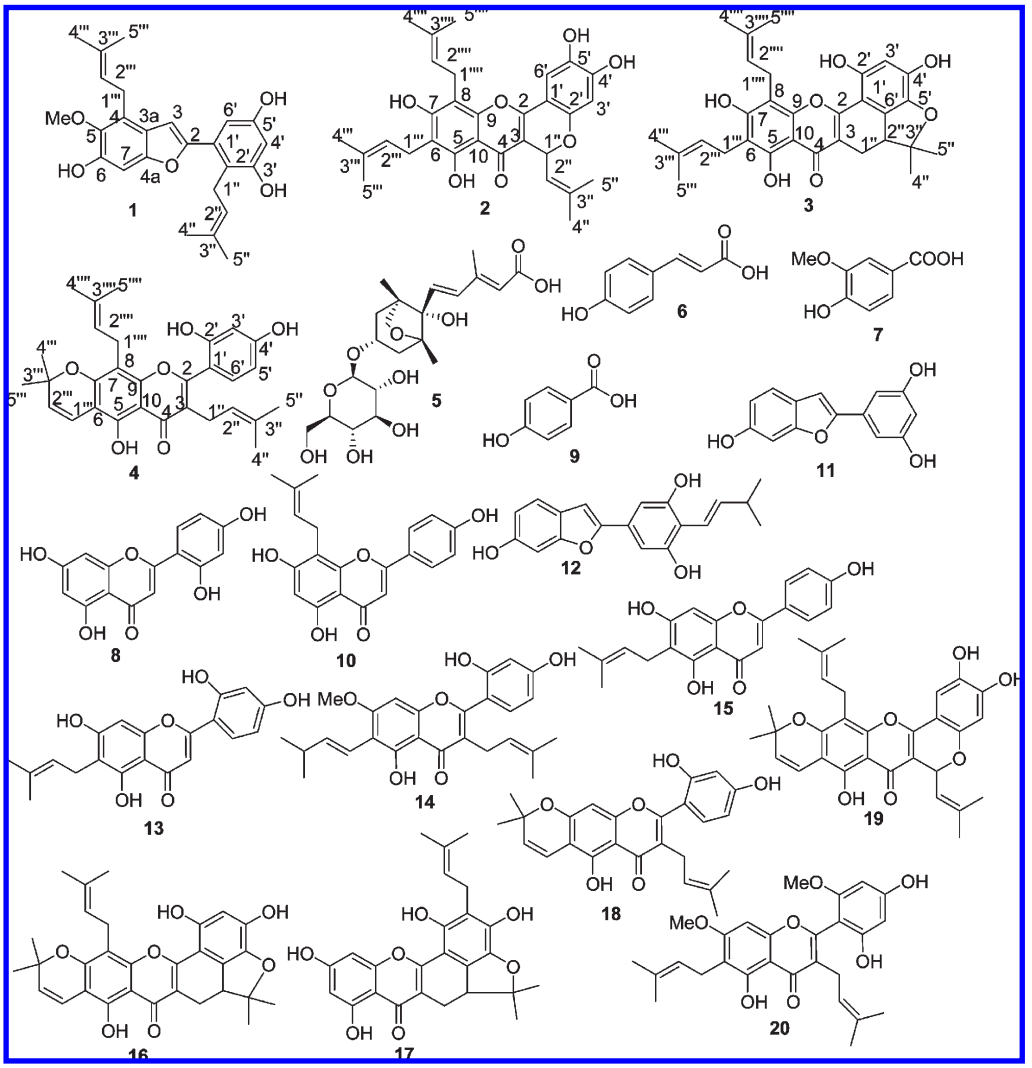

Figure 2. Structures of compounds isolated from the twigs of *A. heterophyllus*.

on C-3 linked by a C-1″ oxygen to C-2′ of the B-ring appeared at δ 6.09 (H-1″), 5.43 (H-2″), and 1.93 and 1.69 (H-4″, 5″). The other two isoprenyl groups, one on C-6 and one on C-8, appeared at δ 5.18 and 5.24 (H-2‴, H-2⁗), 3.34 and 3.56 (H-1‴, H-1⁗), 1.76, 1.85, 1.64, and 1.68 (H-4‴, H-4⁗, H-5‴,5⁗). The proposed structure was further confirmed by the long-range correlations between H-1″ and C-2, C-3, C-4, and C-2′, between H-1‴ and C-5, C-6, and C-7, and between H-1⁗ and C-7, C-8, and C-9 in the HMBC spectrum (**Figure 3**). From these data, the structure of **2** was proposed as the structure we show in **Figure 2** and named artoheterophyllin B.

The molecular formula of compound **3** was deduced as $C_{30}H_{32}O_7$ on the basis of ESI-MS (*m*/*z* 503.3 [M − H]$^-$) and NMR data. The ^{13}C NMR spectrum revealed the presence of 30 carbons, including 6 methyl groups and a carbonyl group (δ 182.6), assignable to an isoprenylated flavonoid. The ^{1}H NMR spectrum showed the signals for two methyl groups at δ 1.32 and 1.65 (each 3H, s) and an AB spin system at δ 2.36, 3.18, and 3.37, assignable to a isopentanyl group located at the C-3 position and, similar to the arrangement found in a related compound, artonin A (*15*). The ^{1}H NMR spectrum also indicated the presence of two isoprenyl groups [δ 3.36 (2H), 3.47 (1H), 3.75 (1H) (H-1‴,1⁗), 5.20, 5.28 (H-2‴,2⁗), 1.65, 1.67, 1.79 (H-4‴,4⁗, 5‴,5⁗)]. Moreover, an aromatic singlet at δ 6.24 was consistent with a 1,2,4,5,6-pentasubstituted B-ring. In the HMBC experiments (**Figure 3**), the protons at δ 2.36 and 3.18 (H-1″) had correlations with carbons at δ 182.6 (C-4), 112.6 (C-3), and 162.7 (C-2), confirming the presence of one isopentanyl group at the C-3 position. The protons at δ 3.36, 3.47, and 3.75 (H-1‴,1⁗) had correlations with carbons δ 157.9 (C-5), 160.4 (C-7), and 153.9 (C-9), confirming the locations of two isoprenyl groups, one at C-6 and one at C-8 of the A ring. Further evidence for the proposed structure of **3** came from comparison of its ^{13}C NMR spectrum with that of a related compound, artonin A, and from HSQC and HMBC data. Thus, compound **3** was assigned as shown in **Figure 2** and named artoheterophyllin C.

Compound **4** showed a molecular ion peak at *m*/*z* 487.3 [M−H]$^-$ in negative ESI-MS spectrum, assignable to a molecular formula of $C_{30}H_{32}O_6$. The ^{1}H and ^{13}C NMR spectra of **4** looked very similar to those of cudraflavone B (*16*), except for the signals for an additional γ,γ-dimethylallyl group. In the ^{1}H NMR spectrum, the B ring exhibited the usual signals of 1,2,4-trisubtitution with H-3′, H-5′, and H-6′ at δ 6.30 (1H, d, J = 2.2 Hz), 6.39 (1H, dd, J = 8.3, 2.2 Hz), and 7.07 (1H, d, J = 8.3 Hz), respectively. The ^{1}H NMR of **4** clearly showed the signals for two isoprenyl groups [δ 3.10 (H-1″), 5.12 (H-2″), 1.28, 1.58 (H-4″,5″), 3.30 (H-1⁗), 5.13 (H-2⁗), 1.60 (H-4⁗,5⁗)] and one 2, 2-dimethylpyran group [5.68 (d, J = 10.0 Hz, H-1‴), 6.70 (d, J = 10.0 Hz, H-2‴), 1.40 (6H, s, H-4‴, 5‴)]. In the HMBC experiments (**Figure 3**), the protons at δ 3.10 (H-1″) had correlations with carbons at δ 184.5 (C-4), 122.0 (C-3), and 164.0 (C-2), indicating the presence of one isoprenyl group at the C-3 position. The protons at δ 3.30 (H-1⁗)

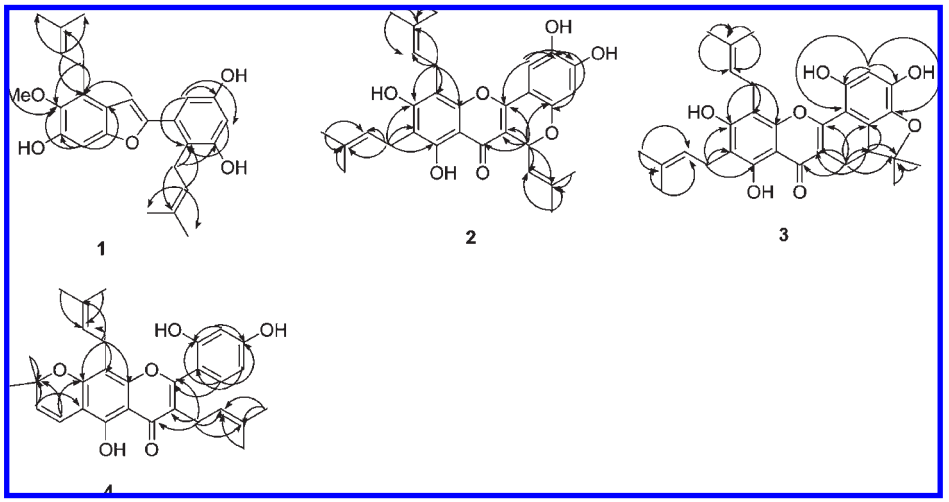

Figure 3. Key HMBC correlations for compounds **1**−**4**.

Table 4. Tyrosinase Inhibitory Activities of Compounds from the Twigs of *A. heterophyllus*

	tyrosinase inhibition activity (%)					
compd	100 μg/mL	50 μg/mL	25 μg/mL	10 μg/mL	5 μg/mL	IC_{50} (μM)
1	turbid	turbid	10	4.8	4.3	
2	turbid	turbid	turbid	NA[a]	NA	
3	turbid	turbid	turbid	14.8	4.8	
4	turbid	turbid	13.8	9.8	4.5	
5	NA	NA	NA	NA	NA	
6	50.3	42.0	33.5	26.3	8.8	603.7
7	NA	NA	NA	NA	NA	
8	100	98.5	98.0	97.3	96.8	0.9
9	17.0	7.5	7.0	3.5	3.5	
10	turbid	turbid	13.8	13.0	12.3	
11	96.3	82.5	66.0	44.8	33.8	57.9
12	95.5	92.5	51.8	25.8	14.0	77.4
13	98.8	93.5	91.8	88.3	86.7	1.7
14	turbid	NA	NA	NA	NA	
15	turbid	turbid	13.5	12.8	11.2	
16	turbid	turbid	turbid	NA	NA	
17	turbid	turbid	35.0	15.0	11.5	
18	turbid	turbid	turbid	8.3	6.5	
19	turbid	turbid	turbid	14.8	NA	
20	turbid	turbid	15.5	7.5	7.5	
kojic acid	98.3	97.8	92.3	70.5	43.5	47.2

[a] NA, no activity.

had correlations with carbons at δ 157.9 (C-7) and 156.7 (C-9), confirming another isoprenyl group at C-8 of the A ring. Further evidence for the structure assigned to **4** was obtained by comparing its ^{13}C NMR spectrum with a related compound, cudraflavone B, and by HSQC and HMBC experiments. Thus, compound **4** was assigned the structure shown in **Figure 2** and named artoheterophyllin D.

The known compounds were identified as dihydrophaseic acid 4′-*O*-β-D-glucopyranoside (**5**) (*17*), *p*-counmaric acid (**6**) (*18*), vanillic acid (**7**) (*19*), norartocarpetin (**8**) (*20*), 4-hydroxybenzoic acid (**9**) (*21*), licoflavone C (**10**) (*22*), moracin M (**11**) (*23*), (*E*)-5-(6-hydroxybenzofuran-2-yl)-4-(3-methylbut-l-enyl)benzene-1,3-diol (**12**) (*24*), artocarpesin (**13**) (*20*), artocarpin (14) (*25*), 6-prenyl-4′,5,7-trihydroxyflavone (**15**) (*26*), artonin A (**16**) (*14*), artonin J (**17**) (*27*), cudraflavone B (**18**) (*16*), cycloheterophyllin (**19**) (*28*), and 2-(2,4-dihydroxy-6-methoxyphenyl)-5-hydroxy-7-methoxy-6-(3-methyl-l-buten-l-yl)-3-(3-methyl-2-buten-l-yl)-4*H*-1-benzopyran-4-one (**20**) (*29*) by comparing the MS, ^1H NMR, and ^{13}C NMR data with those of reference compounds.

Tyrosinase inhibitory activity of compounds **1**−**20** was examined using mushroom tyrosinase with L-tyrosine as a substrate. Each compound was assayed over a range of concentrations (**Table 4**). Results showed that norartocarpetin (**8**) and artocarpesin (**13**), which were also present in the wood of *A. heterophyllus* (*10*), had good tyrosinase inhibitory activity. Moracin M (**11**) and (*E*)-5-(6-hydroxybenzofuran-2-yl)-4-(3-methylbut-l-enyl)benzene-1,3-diol (**12**) showed moderate inhibitory activities against mushroom tyrosianse, but both were weaker than kojic acid. Other compounds showed rather weak tyrosinase inhibitory activities, and further increase in concentration ($\geq$50 μg/mL) to determine their IC_{50} led to precipitation of the compounds in the test solvent system.

In conclusion, chromatographic, and spectroscopic analyses demonstrated that the chemical profiles of the wood and twigs of *A. heterophyllus* were different. Its twigs contained some novel natural products that were not identified from the wood. However, it was found that similar compounds in the wood and twig contributed to their tyrosinase inhibitory activities, and norartocarpetin and artocarpesin were identified as the most active inhibitors in the twigs of *A. heterophyllus*.

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