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Chemical Composition and Antioxidant Activity of Phenolic Compounds from Wild and Cultivated *Sclerocarya birrea* (Anacardiaceae) Leaves

ALESSANDRA BRACA,[†] MATTEO POLITI,[†] ROKIA SANOGO,[#] HABY SANOU,[‡] IVANO MORELLI,[†] COSIMO PIZZA,[§] AND NUNZIATINA DE TOMMASI*,[§]

Dipartimento di Chimica Bioorganica e Biofarmacia, Università di Pisa, Via Bonanno 33, 56126 Pisa, Italy; Departement Medecine Traditionelle (DMT), INRSP, B.P. 1746, Bamako, Mali; Institut d'Economie Rurale (IER), B.P. 258, Bamako, Mali; and Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA), Italy

A quantitative study of the phenolic constituents of wild and cultivated leaves of *Sclerocarya birrea* (Anacardiaceae) was carried out by HPLC-UV/PDA and LC-MS. Phytochemical analysis of the methanol extract of wild plants led to the isolation of one new flavonol glycoside, quercetin 3-*O*-α-L-(5″-galloyl)-arabinofuranoside (**1**), and eight known phenolic compounds; two epicatechin derivatives were also isolated from the same extract of the cultivated species. The antioxidant activity of all isolated compounds was determined by measuring free radical scavenging effects using the Trolox equivalent antioxidant capacity assay and the coupled oxidation of β-carotene and linoleic acid (autoxidation assay).

KEYWORDS: ***Sclerocarya birrea*; phenolic compounds; HPLC-UV/PDA; LC-MS; NMR; antioxidant activity**

INTRODUCTION

Sclerocarya birrea (A. Rich.) Hochst (Anacardiaceae family), known in the Bambara language of Mali by the common local name of "ngunan", is used in traditional Malian medicine for the treatment of several diseases: the leaves and the pulp of fruit are used for hypertension; the leaves are used against diabetes, dysentery, snake and scorpion bites, malaria, and inflammations; the plant is also utilized as a tonic, and the fruits are often fermented to give a refreshing drink (*1*). Previous chemical and pharmacological studies on the bark have reported the antidiarrheal activity of the decoction (*2*) and the isolation of a procyanidin compound (*3*) and of (−)-epicatechin-3-galloyl ester (*4*). The leaves of the plant were not chemically previously investigated, although their extract exhibited activity with Ca^{2+}-mobilizing systems in muscle cells (*5*).

In Mali, the Traditional Medicine Department (DMT), an official structure connected with the National Institute of Research in Public Health (INRSP) of the Health Minister, is a collaborating center of the World Health Organization (WHO) for research in traditional medicine. According to the policy of WHO, one of the primary objectives of the DMT is to establish a mechanism to ensure that traditional medicine may be complementary to conventional medicines, assuming that drugs can be produced from local resources and mainly from medicinal plants. The main activity of the DMT is the research and development of improved traditional medicines (ITM). Among these preparations, "diabetisane" is an ITM obtained from the decoction of the leaves of *S. birrea* and is used for the treatment of diabetes and related diseases (*6*). The leaves are usually collected from adult plants >10 years old, and this could provoke environmental damage. To reduce the depletion of wild plants the Institut d'Economie Rurale (IER) is carrying out experimental cultivations of *S. birrea* with the aim of using the young cultivated species (2−3 years old) instead of wild plants to produce ITM "diabetisane".

The present study was carried out to compare the quali-quantitative composition in phenolic compounds of polar extracts of wild and cultivated *S. birrea* leaves. The target isolation of phenolic derivatives was obtained from both species leaves with the aim of evaluating their antioxidant activity by measuring free radical scavenging effects using the Trolox equivalent antioxidant capacity (TEAC) assay and the coupled oxidation of β-carotene and linoleic acid (autoxidation assay). A new minor flavonol glycoside, quercetin 3-*O*-α-L-(5″-galloyl)-arabinofuranoside (**1**), was also identified.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and at 150.86 MHz for

* Corresponding author (telephone+39-089-962647; fax +39-089-962828; e-mail detommasi@unisa.it).
[†] Università di Pisa.
[#] INRSP, Mali.
[‡] IER, Mali.
[§] Università di Salerno.

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^{13}C, using the UXNMR software package was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, *J*, are in hertz. DEPT ^{13}C, 1D-TOCSY, ^{1}H−^{1}H DQF-COSY, ^{1}H−^{13}C HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. Column chromatographies were performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden); HPLC separations were conducted on a Waters 600E chromatograph, equipped with a Waters 996 photodiode array detector (PDA) (Waters, Milford, MA).

Plant Material. The leaves of wild *S. birrea* were collected in Bamako region in October 2000 and were identified by Prof. N'Golo Diarra of the DMT, Bamako, Mali, where a voucher specimen is deposited (voucher no. DMT106); the cultivated leaves of *S. birrea* were obtained from the IER cultivated fields in October 2001.

On-line Analyses. HPLC-UV/PDA experiments were performed on a Lichrospher C_{18} (5 μm) column (LichroCART) (250 × 4.6 mm) equipped with a PDA. HPLC-ESI/MS analyses were performed using the same HPLC column with a ThermoFinnigan Surveyor LC pump coupled with a Finnigan LCQ Advantage ion trap mass (San Jose, CA). For both HPLC analyses, 400 μg of the crude extracts dissolved in MeOH was injected. The flow rate was 1 mL min^{-1} with the following MeOH/H_2O gradient: 0 min, MeOH/H_2O (5:95); 50 min, MeOH 100% (maintained for 10 min). For LC-MS analyses the postcolumn split ratio was 10:2. PDA data were recorded with a 210−500 nm range, with 254 nm as the detection wavelength. All LC-MS analyses were performed with an electrospray ionization (ESI) interface in negative ion modes. The ionization conditions were optimized, and the following parameters were retained: capillary temperature, 280 °C; sheath gas flow, 70 psi; source voltage, 4.00 kV; scan range, 150−1000 amu.

Extraction and Isolation Procedures of Compounds 1−11. The air-dried powdered leaves of wild *S. birrea* (386 g) were defatted with petroleum ether and successively extracted for 48 h with $CHCl_3$, $CHCl_3$/MeOH (9:1), and MeOH, by exhaustive maceration (3 × 2 L), to give 8.0, 20.0, 4.3, and 22.5 g of the respective residues. Part of the methanol extract (2.4 g) was chromatographed on Sephadex LH-20, using MeOH as eluent (flow rate = 1 mL min^{-1}), to obtain 10 major fractions, together with pure compounds **2** and **3**. Fraction 4 (44 mg) was purified by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate = 2.0 mL min^{-1}) with MeOH/H_2O (3:7) to yield pure compound **9** (t_R = 8 min, 10.0 mg). Fraction 6 (350 mg) was chromatographed on RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate = 2.0 mL min^{-1}) with MeOH/H_2O (4.5:5.5) to obtain pure compounds **7** (t_R = 17 min, 3.0 mg), **4** (t_R = 23 min, 5.0 mg), **6** (t_R = 27 min, 7.5 mg), **8** (t_R = 31 min, 3.2 mg), and **5** (t_R = 35 min, 2.0 mg). Fraction 9 (40 mg) was chromatographed on RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate = 2.0 mL min^{-1}) with MeOH/H_2O (45:55) to give pure compound **1** (t_R = 18 min, 5.0 mg).

The air-dried powdered leaves of cultivated *S. birrea* (100 g) were defatted with petroleum ether and successively extracted for 48 h with $CHCl_3$, $CHCl_3$/MeOH (9:1), and MeOH, by exhaustive maceration (3 × 1 L), to give 1.8, 4.5, 0.8, and 8.0 g of the respective residues. Part of the methanol extract (2.5 g) was chromatographed on Sephadex LH-20, using MeOH as eluent (flow rate = 1 mL min^{-1}) to obtain pure compounds **10** and **11**.

Quercetin 3-*O*-α-(5″-galloyl)arabinofuranoside (1) was obtained as a yellow amorphous powder: [α] +9.3° (*c* 0.05, MeOH); ESI-MS, *m/z* 585 [M − H]$^-$, 433 [M − H − 152]$^-$, 301 [M − H − 152 − 132]$^-$; ^{1}H NMR and ^{13}C NMR, see **Table 1**. Eelemental analysis: C, 55.26%; H, 3.79%; O, 40.95%. Calcd for $C_{27}H_{22}O_{15}$: C, 55.30%; H, 3.78%; O, 40.92%.

Quercetin 3-*O*-β-D-(6″-galloyl)glucopyranoside (2) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 263, 354 nm; ESI-MS, *m/z* 615 [M−H]$^-$, 463 [M − H − 152]$^-$, 301 [M − H − 152 − 162]$^-$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 4.19 (1H, dd, *J* = 12.0, 5.0 Hz, H-6″a), 4.23 (1H, dd, *J* = 12.0, 3.0 Hz, H-6″b), 5.41 (1H, d, *J* = 7.8 Hz, H-1″), 6.17 (1H, d, *J* = 1.8 Hz, H-6), 6.36 (1H, d, *J* = 1.8 Hz, H-8), 6.71 (1H, d, *J* = 8.0 Hz, H-5′), 6.89 (2H, s, H-2‴ and H-6‴), 7.42 (1H, d, *J* = 2.0 Hz, H-2′), 7.57 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 63.2 (C-6″), 69.6 (C-4″), 74.1 (C-2″), 74.2 (C-5″), 76.4 (C-3″), 93.7 (C-8), 98.8 (C-6), 101.4 (C-1″), 104.0 (C-10), 108.7 (C-2‴ and C-6‴), 115.4 (C-2′), 115.8 (C-5′), 119.4 (C-1‴), 121.1 (C-1′), 122.0 (C-6′), 133.4 (C-3), 138.5 (C-4‴), 144.9 (C-3′), 145.5 (C-3‴ and C-5‴), 148.5 (C-4′), 156.4 (C-2), 156.5 (C-9), 161.3 (C-5), 164.3 (C-7), 165.8 (COO), 177.4 (C-4) (*7*).

Table 1. ^{1}H and ^{13}C NMR Data (600 MHz, CD_3OD) and HMBC Correlations of Compound **1**[a]

	δ_H	δ_C	HMBC
2		158.3	7.47, 7.51
3		136.0	5.50
4		179.0	
5		163.2	6.21
6	6.21 d (1.5)	100.1	
7		166.1	6.39
8	6.39 d (1.5)	95.2	6.21
9		159.0	6.39
10		105.7	6.21, 6.39
1′		122.0	7.47, 7.51
2′	7.51 d (1.8)	117.0	7.47
3′		145.8	6.91, 7.51
4′		149.5	6.91, 7.47
5′	6.91 d (8.0)	116.4	7.47
6′	7.47 dd (8.0, 1.8)	123.0	6.91, 7.51
3-*O*-ara-1″	5.50 br s	109.6	
2″	4.42 dd (3.0, 1.0)	83.7	
3″	3.97 br d (4.0)	79.2	
4″	3.99 m	84.6	
5″a	4.15 dd (12.0, 5.7)	64.6	
5″b	4.22 dd (12.0, 3.0)		
galloyl 1‴		122.5	7.02
2‴	7.02 s	110.3	
3‴		146.4	7.02
4‴		139.3	7.02
5‴		146.4	
6‴	7.02 s	110.3	
COO		169.0	4.15, 4.22

[a] Coupling pattern; and coupling constant (*J* in hertz) is in parentheses.

Quercetin 3-*O*-β-D-(6″-galloyl)galactopyranoside (3) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 262, 353 nm; ESI-MS, *m/z* 615 [M − H]$^-$, 463 [M − H − 152]$^-$, 301 [M − H − 152 − 162]$^-$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 4.04 (1H, dd, *J* = 12.0, 5.0 Hz, H-6″a), 4.08 (1H, dd, *J* = 12.0, 3.0 Hz, H-6″b), 5.36 (1H, d, *J* = 7.6 Hz, H-1″), 6.18 (1H, d, *J* = 1.8 Hz, H-6), 6.39 (1H, d, *J* = 1.8 Hz; H-8), 6.81 (1H, d, *J* = 8.0 Hz, H-5′), 6.88 (2H, s, H-2‴ and H-6‴), 7.51 (1H, d, *J* = 2.0 Hz, H-2′), 7.65 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 62.0 (C-6″), 67.8 (C-4″), 71.1 (C-2″), 72.8 (C-5″), 72.9 (C-3″), 93.5 (C-8), 98.7 (C-6), 102.2 (C-1″), 103.9 (C-10), 108.6 (C-2‴ and C-6‴), 115.2 (C-2′), 116.0 (C-5′), 119.1 (C-1‴), 121.1 (C-1′), 121.9 (C-6′), 133.6 (C-3), 138.5 (C-4‴), 144.8 (C-3′), 145.5 (C-3‴ and C-5‴), 148.5 (C-4′), 156.3 (C-2), 156.4 (C-9), 161.2 (C-5), 164.2 (C-7), 165.5 (COO), 177.4 (C-4) (*7*).

Quercetin 3-*O*-α-L-rhamnopyranoside (4) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 255, 349 nm; ESI-MS, *m/z* 447 [M − H]$^-$, 301 [M − H − 146]$^-$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 0.79 (3H, d, *J* = 6.0 Hz, H-6″), 5.24 (1H, d, *J* = 1.8 Hz, H-1″), 6.19 (1H, d, *J* = 1.8 Hz, H-6), 6.38 (1H, d, *J* = 1.8 Hz, H-8), 6.85 (1H, d, *J* = 8.0 Hz, H-5′), 7.24 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 7.28 (1H, d, *J* = 2.0 Hz, H-2′); ^{13}C NMR (CD_3OD, 150 MHz) δ 17.6 (C-6″), 70.1 (C-5″), 70.4 (C-3″), 70.2 (C-2″), 71.2 (C-4″), 93.7 (C-8), 98.8 (C-6), 101.9 (C-1″), 104.1 (C-10), 115.3 (C-2′), 115.9 (C-5′), 120.8 (C-1′), 121.2 (C-6′), 134.3 (C-3), 145.3 (C-3′) 148.5 (C-4′), 156.5 (C-2), 157.4 (C-9), 161.3 (C-5), 164.3 (C-7), 177.8 (C-4) (*7*).

Kaempferol 3-*O*-β-D-(6″-galloyl)glucopyranoside (5) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 264, 350 nm; ESI-MS, *m/z* 599 [M − H]$^-$, 447 [M − H − 152]$^-$, 285 [M − H − 152 − 162]$^-$; ^{1}H NMR (CD_3OD, 600 MHz) δ 5.21 (1H, d, *J* = 7.7 Hz, H-1″), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 6.35 (1H, d, *J* = 2.0 Hz, H-8), 6.73 (2H, d, *J* = 8.0 Hz, H-3′ and H-5′), 6.94 (2H, s, H-2‴ and H-6‴), 7.94 (2H, d, *J* = 8.0 Hz, H-2′ and H-6′); ^{13}C NMR (CD_3OD, 150 MHz)

δ 64.3 (C-6″), 71.5 (C-4″), 75.9 (C-2″), 75.8 (C-5″), 78.1 (C-3″), 95.0 (C-8), 100.0 (C-6), 104.4 (C-1″), 105.7 (C-10), 110.3 (C-2‴ and C-6‴), 132.2 (C-2′ and C-6′), 116.1 (C-3′ and C-5′), 121.3 (C-1‴), 122.0 (C-1′), 136.0 (C-3), 139.3 (C-4‴), 146.4 (C-3‴ and C-5‴), 161.5 (C-4′), 158.5 (C-2), 159.0 (C-9), 163.0 (C-5), 166.0 (C-7), 168.2 (COO), 179.4 (C-4) (*8*).

Quercetin 3-*O*-β-D-glucopyranoside (6) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 262, 293sh, 354 nm; ESI-MS, *m*/*z* 463 [M − H]$^-$, 301 [M − H − 162]$^-$; ^{1}H NMR (CD_3OD, 600 MHz) δ 5.12 (1H, d, *J* = 8.0 Hz, H-1″), 6.16 (1H, d, *J* = 1.8 Hz, H-6), 6.34 (1H, d, *J* = 1.8 Hz, H-8), 6.85 (1H, d, *J* = 8.0 Hz, H-5′), 7.60 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 7.66 (1H, d, *J* = 2.0 Hz, H-2′); ^{13}C NMR (CD_3OD, 150 MHz) δ 62.4 (C-6″), 75.4 (C-2″), 71.2 (C-4″), 78.0 (C-5″), 78.3 (C-3″), 94.7 (C-8), 99.9 (C-6), 102.5 (C-1″), 105.6 (C-10), 116.2 (C-2′), 117.5 (C-5′), 122.8 (C-1′), 123.1 (C-6′), 135.6 (C-3), 145.9 (C-3′), 148.5 (C-4′), 158.3 (C-2), 158.6 (C-9), 162.9 (C-5), 165.9 (C-7), 179.4 (C-4) (*7*).

Myricetin 3-*O*-α-L-rhamnopyranoside (7) was obtained as an orange amorphous powder: UV (MeOH) λ_{max} 262, 301sh, 351 nm; ESI-MS, *m*/*z* 463 [M − H]$^-$, 317 [M − H − 146]$^-$; ^{1}H NMR (200 MHz, CD_3OD) δ 0.98 (3H, d, *J* = 6.5 Hz, H-6″), 3.27−3.89 (4H, m, sugar protons), 5.32 (1H, d, *J* = 1.8 Hz, H-1″), 6.12 (1H, d, *J* = 2.0 Hz, H-6), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 7.08 (2H, s, H-2′ and 6′); ^{13}C NMR (50 MHz, DMSO-d_6) δ 17.3 (C-6″), 70.5 (C-5″), 70.8 (C-2″), 71.0 (C-3″), 71.8 (C-4″), 94.5 (C-8), 99.6 (C-6), 102.4 (C-1″), 105.2 (C-10), 109.4 (C-2′ and C-6′), 121.7 (C-1′), 134.4 (C-3), 137.2 (C-4), 146.8 (C-3′ and C-5′), 157.5 (C-9), 158.2 (C-2), 163.2 (C-5), 165.4 (C-7), 178.0 (C-4) (*9*).

Kaempferol 3-*O*-α-L-rhamnopyranoside (8) was obtained as a yellow amorphous powder: UV (MeOH) λ_{max} 263, 343 nm; ESI-MS, *m*/*z* 431 [M − H]$^-$, 285 [M − H − 146]$^-$; ^{1}H NMR (200 MHz, CD_3OD) δ 1.01 (3H, d, *J* = 6.5 Hz, H-6″), 3.27−4.20 (4H, m, sugar protons), 5.43 (1H, d, *J* = 1.8 Hz, H-1″), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.94 (2H, d, *J* = 8.4 Hz, H-3′ and H-5′), 7.78 (2H, d, *J* = 8.4 Hz, H-2′ and 6′) (*10*).

Gallic acid (9) was obtained as white needles: ^{1}H NMR (200 MHz, CD_3OD) δ 7.04 (2H, s, H-2 and H-6); ^{13}C NMR (50 MHz, CD_3OD) δ 110.3 (C-2 and C-6), 122.5 (C-1), 139.5 (C-4), 146.3 (C-3), 171.0 (COOH) (*11*).

(−)-Epicatechin 3-*O*-galloyl ester (10) was obtained as a red amorphous powder: UV (MeOH) λ_{max} 276 nm; ESI-MS, *m*/*z* 441 [M − H]$^-$, 289 [M − H − 152]$^-$; ^{1}H NMR (CD_3OD, 600 MHz) δ 2.84 (1H, br d, H-4a), 3.00 (1H, dd, *J* = 17.6, 4.4 Hz, H-4b), 5.54 (1H, br s, H-3), 5.98 (2H, br s, H-6 and H-8), 6.69 (1H, d, *J* = 8.0 Hz, H-5′), 6.82 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 6.90 (1H, d, *J* = 2.0 Hz, H-2′), 6.98 (2H, s, H-2″ and H-6″); ^{13}C NMR (CD_3OD, 150 MHz) δ 26.9 (C-4), 70.0 (C-3), 78.6 (C-2), 95.9 (C-8), 96.7 (C-6), 99.4 (C-10), 110.2 (C-2″ and C-6″), 115.1 (C-2′), 116.0 (C-5′), 119.4 (C-6′), 121.4 (C-1″), 131.5 (C-1′), 139.8 (C-4″), 145.9 (C-3′ and C-4′), 146.3 (C-3″ and C-5″), 157.3 (C-5), 157.8 (C-7 and C-9), 167.6 (COO) (*4*).

(−)-Epigallocatechin 3-*O*-galloyl ester (11) was obtained as a red amorphous powder: UV (MeOH) λ_{max} 274 nm; ESI-MS, *m*/*z* 457 [M − H]$^-$, 305 [M − H − 152]$^-$; ^{1}H NMR (CD_3OD, 200 MHz) δ 2.91 (1H, dd, *J* = 17.4, 2.2 Hz, H-4a), 3.04 (1H, dd, *J* = 17.4, 4.6 Hz, H-4b), 5.56 (1H, br s, H-3), 6.03 (1H, d, *J* = 2.3 Hz, H-8), 6.06 (1H, d, *J* = 2.3 Hz, H-6), 6.62 (2H, s, H-2′ and H-6′), 7.03 (2H, s, H-2″ and H-6″); ^{13}C NMR (CD_3OD, 200 MHz) δ 26.6 (C-4), 69.3 (C-3), 78.1 (C-2), 95.9 (C-8), 96.5 (C-6), 99.1 (C-10), 106.8 (C-2′ and C-6′), 110.0 (C-2″ and C-6″), 121.9 (C-1″), 130.8 (C-1′), 133.2 (C-4′), 138.8 (C-4″), 145 (C-3″ and C-5″), 146.3 (C-3′ and C-5′), 157.1 (C-9), 157.5 (C-5), 157.8 (C-7), 166.1 (COO) (*12*).

TEAC Assay. Pure compounds were tested by using the TEAC assay. The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonate) (ABTS$^{\bullet+}$) measured by spectrophotometric analysis (*13*). ABTS$^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The ABTS$^{\bullet+}$ solution was then diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Samples were diluted with methanol to produce solutions of 0.3, 0.5, 1, 1.5, and 2 mM concentration. The reaction was initiated by the addition of 1 mL of diluted ABTS to 10 μL of each sample solution. Determinations were repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of concentration compound or standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co., Gillingham, Dorset, U.K.). The antioxidant activities of compounds **1**−**11** are expressed as TEAC values in comparison with TEAC activity of reported reference compound quercetin (*13*). The TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant compound under investigation.

Autoxidation of β-Carotene Assay. Oxidation of linoleic acid was measured according to the method described by Pratt (*14*). Quantities of linoleic acid (20 mg) and Tween 20 (200 mg) were placed in a flask, and a solution of 2 mg of β-carotene in 10 mL of $CHCl_3$ was added. After removal of $CHCl_3$, 50 mL of distilled water saturated with oxygen for 30 min was added. Aliquots (200 μL) of each compound, dissolved in ethanol to a 15 μg/mL solution, were added to each flask with shaking. Samples without test compounds were used as blanks, and a sample with 2,6-di-*tert*-butyl-4-methoxyphenol (BHT, Aldrich Chemical Co.) was used as a control substance. Samples were subjected to oxidation by placing in an oven at 50 °C for 3 h. The absorbance was read at 470 nm at regular intervals. The antioxidant activity (AA) was calculated with the equation

$$AA = [1 - (A_0 - A_t)]/(A_{00} - A_{0t}) \times 100$$

where A_0 = absorbance at the beginning of the incubation, with test compound; A_t = absorbance at the time *t*, with test compound; A_{00} = absorbance at the beginning of the incubation, without test compound; and A_{0t} = absorbance at the time *t*, without test compound.

RESULTS

HPLC-UV/PDA and HPLC-ESI/MS Analyses. To evaluate the possible use of the cultivated *S. birrea* instead of wild plant as source of Malian ITM, the chemical compositions of both plant leaves were studied using the on-line methods LC-UV/PDA and LC-MS. HPLC-UV/PDA profiles showed a very close chemical composition of both MeOH residues on the basis of the retention time (t_R) of the detected compounds. In fact, peaks D (t_R = 27.1 min), E (t_R = 27.8 min), F (t_R = 29.0 min), G (t_R = 29.8 min), H (t_R = 30.8 min), and I (t_R = 33.3 min) were present in both extracts as shown in **Figure 1**. Besides, peak A (t_R = 6.9 min) was detected in the wild plant (**Figure 1A**), whereas for the cultivated species peaks B and C were observed at t_R = 20.2 and 23.6 min, respectively (**Figure 1B**). The classes of secondary metabolites were recognized on the basis of their typical UV spectra and by comparison with literature data (*15*). Peaks D−I, detected in both extracts, had similar UV spectra with their absorption maxima (λ_{max}) between 255 and 265 and 345−355 nm (**Figure 1A**). These UV spectra were typical of flavonol compounds (*15*). The single band near 270 nm observed for peaks B and C in the chromatogram of the methanol extract of the cultivated plant (**Figure 1B**) seemed to be due to a nonconjugated aromatic ring. Analysis of the literature data (*4*) showed the presence of catechin derivatives in the roots of *S. birrea*; this class of secondary metabolite presented the same UV spectra of peaks B and C (*16*). LC-MS analyses were performed on both extracts to improve the on-line data of each compound detected and to confirm the hypothesized structures from UV spectra. LC-MS spectra of compounds B and C showed their pseudomolecular ions [M − H]$^-$ at *m*/*z* 457 and 441, respectively (**Table 2**); a loss of 152 mass units, probably due to a galloyl group, was observed in both compounds. Results obtained from UV and MS data allowed their partial identification as (−)-epicatechin gallate and (−)-epigallocatechin gallate

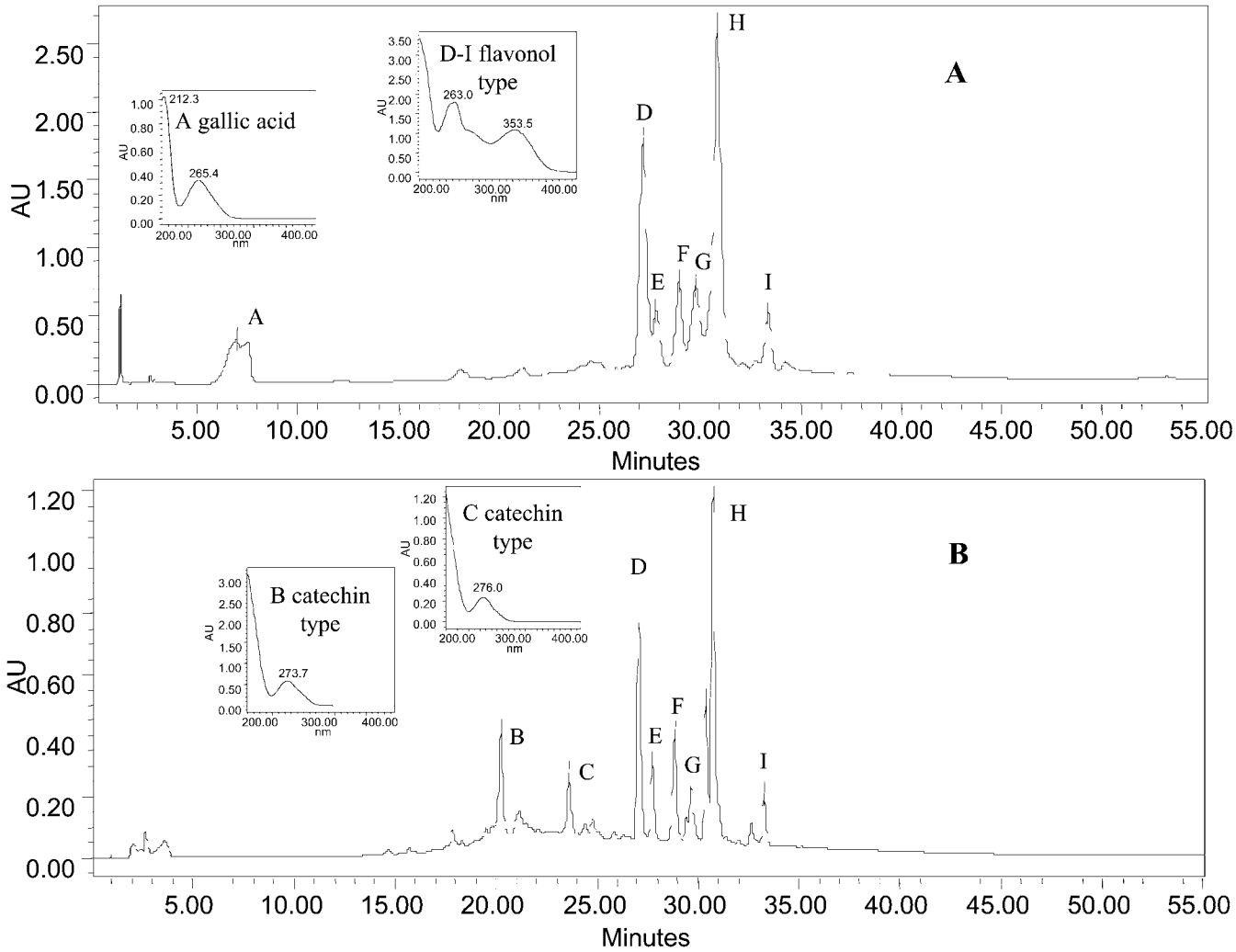

Figure 1. HPLC analytical data of methanol extracts of wild (A) and cultivated (B) *S. birrea* leaves monitored at 254 nm. Peaks: A = **9**; B = **11**; C = **10**; D = **2** and **3**; E = **7**; F = **6**; G = **5**; H = **4**; I = **8**. (For chromatographic conditions see Materials and Methods.)

Table 2. Ions of Structural Significance and Retention Time Data of LC-MS Analyses of MeOH Extracts of Wild and Cultivated *S. birrea* Plants

t_R (min)	label	ESI neg $(M-H)^-$	fragments *m/z*	losses
6.9	A (**9**)			
20.2	B (**11**)	456.9	305.4	152
23.6	C (**10**)	440.9	289.3	152
27.1	D (**2**, **3**)	615.1	462.8, 301.1	152, 162
27.8	E (**7**)	463.1	317.1	146
28.9	F (**6**)	463.1	301.1	162
29.8	G (**5**)	599.1	447.1, 285.2	152, 162
30.8	H (**4**)	447.1	301.0	146
33.3	I (**8**)	431.1	285.1	146

(*12*). The fragments observed in LC-MS analysis of flavonoids D−I allowed us to suppose the presence of different sugar moieties in the molecules; for peaks D and G a loss of a galloyl group was also observed (**Table 2**). No pseudomolecular ion was revealed for peak A, which was identified as gallic acid by comparison with an authentic sample.

Isolation and Structure Determination of Compounds 1−11. To confirm the hypothesized structures from the analytical investigations and to evaluate the antioxidant activity of pure phenolic compounds, the isolation procedures of MeOH extracts were undertaken. In total, nine compounds were purified from the wild plant: eight flavonol glycosides, of which one was a new natural product (**1**), and gallic acid (**Figure 2**). Epicatechin derivatives related to peaks B and C were isolated from the cultivated species.

Compound **1** was isolated as a yellow amorphous powder. Its molecular formula was established as $C_{27}H_{22}O_{15}$ by means of ESI-MS, ^{13}C, ^{13}C DEPT NMR, and elemental analysis. Its ESI-MS spectrum showed an $[M - H]^-$ peak at *m/z* 585, together with two major fragments at *m/z* 433 (due to the loss of 152 mass units) and 301 (due to the subsequent loss of 152 and 132 mass units). Analysis of its 600 MHz NMR spectrum suggested a flavonoidic skeleton for compound **1**. The ^1H NMR spectrum indicated a 5,7-dihydroxylated pattern for ring A (two meta-coupled doublets at δ 6.21 and 6.39, $J = 1.5$ Hz) and a 3′,4′-dihydroxylation pattern for ring B [ABX system signals at δ 6.91 (d, $J = 8.0$ Hz), 7.47 (dd, $J = 8.0$, 1.8 Hz), 7.51 (d, $J = 1.8$ Hz)], allowing the aglycon to be recognized as quercetin. One anomeric proton at δ 5.50 and two-proton singlets at δ 7.02 were also identified in this spectrum, suggesting the presence of one saccharidic unit and a galloyl ester moiety. Analysis of the chemical shifts, signal multiplicities, absolute values of the coupling constants, and their magnitude in the ^1H NMR spectrum, as well as ^{13}C NMR data (**Table 1**), indicated the presence of one arabinofuranosyl residue with α-configuration at the anomeric carbon (*17*). The DQF-COSY spectrum together with the 1D TOCSY experiment allowed the assign-

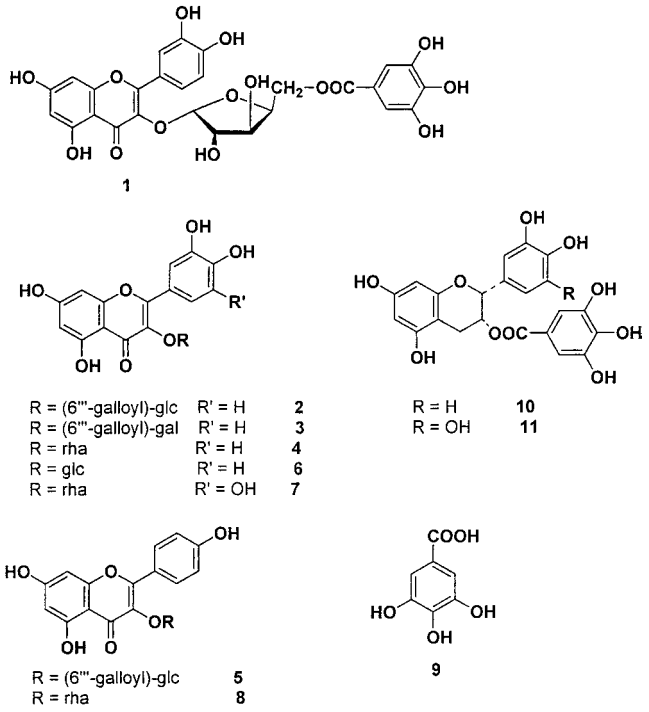

Figure 2. Structures of compounds **1**–**9** isolatd from MeOH extracts of *S. birrea* wild plant and **10**–**11** purified from cultivated species. Their structural elucidation was obtained on the basis of spectroscopic methods (UV, NMR, MS).

ment of the spin systems of the sugar residue. In particular, the lower field shift of the H-5″ (δ 4.15, 4.22) and C-5″ (δ 64.6) of the arabinofuranosyl unit suggested the substitution site of the galloyl residue. An analysis of the C−H long-range coupling connectivities in the HMBC spectrum revealed correlations between δ 5.50 (H-1″) and 136.0 (C-3) and δ 4.15 and 4.22 (H-5″) and 169.0 (COO) of the galloyl residue. Thus, the structure of **1** was determined as quercetin 3-*O*-α-L-(5″-galloyl)-arabinofuranoside.

From the MeOH extract of the wild plant leaves also compounds **2**−**9** were isolated and identified as quercetin 3-*O*-β-D-(6″-galloyl)glucopyranoside (**2**), quercetin 3-*O*-β-D-(6″-galloyl)galactopyranoside (**3**), quercetin 3-*O*-α-L-rhamnopyranoside (**4**), kaempferol 3-*O*-β-D-(6″-galloyl)glucopyranoside (**5**), quercetin 3-*O*-β-D-glucopyranoside (**6**), myricetin 3-*O*-α-L-rhamnopyranoside (**7**), and kaempferol 3-*O*-α-L-rhamnopyranoside (**8**), respectively (**Figure 2**), by comparison with the reported spectral data in the literature (*7*−*10*). Compound **9** was identified as gallic acid (**Figure 2**) by comparison with an authentic sample. From the MeOH extract of the cultivated plants were obtained (−)-epicatechin 3-*O*-galloyl ester (**10**) and (−)-epigallocatechin 3-*O*-galloyl ester (**11**) (**Figure 2**), which were identified by comparison of their spectral data with those reported in the literature (*4*, *12*). Comparison of NMR spectral data and on-line results allowed identification in the UV and MS chromatograms of compounds **2** and **3** as peak D, compound **4** as peak H, compound **5** as peak G, compound **6** as peak F, compound **7** as peak E, compound **8** as peak I, compound **9** as peak A, compound **10** as peak C, and compound **11** as peak B, respectively.

Antioxidant Activity. The antioxidant activity of the isolated compounds **1**−**11** was first tested in TEAC assay. The radical scavenger activity of the tested compounds was expressed as TEAC values; TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant compound under investigation (*13*). TEAC results for compounds **1**−**11** and quercetin, used as reference compound, are summarized in **Table 3**. Among compounds **1**−**11**, (−)-epicatechin 3-*O*-galloyl ester (**10**) and (−)-epigallocatechin 3-*O*-galloyl ester (**11**) have a TEAC value (~3 mM) higher than that of the reference compound quercetin. This result is in agreement with the radical-scavenging activity detected by different methods for catechin derivatives found in green tea and associated with the potent antioxidant property of this herbal infusion (*18*). Quercetin derivatives **2** and **3** showed anyway a good activity (TEAC value ~ 1.8 mM) with respect to the other quercetin glycosides **4** and **6**, probably due to the presence of an esterified galloyl group. All of the other metabolites yielded lower TEAC values comparable to that of the reference compound.

Table 3. Antioxidant Activities of Compounds **1**–**11** in the TEAC and Autoxidation Assays[a]

compound	TEAC value (mM) ± SD[b]	autoxidation assay t = 60 min	autoxidation assay t = 120 min
1	0.68 ± 0.01	12.4	11.0
2	1.92 ± 0.13	32.0	14.0
3	1.56 ± 0.03	20.3	14.2
4	0.85 ± 0.05	26.0	4.8
5	1.60 ± 0.01	24.5	16.5
6	0.76 ± 0.03	26.6	4.0
7	1.30 ± 0.02	21.3	15.0
8	0.75 ± 0.05	18.2	11.0
9	1.06 ± 0.11	28.3	5.0
10	2.79 ± 0.01	35.0	16.0
11	3.01 ± 0.01	42.0	16.0
quercetin	2.77 ± 0.02		
BHT[c]		71.3	64.1

[a] For protocols used, see Materials and Methods. [b] n = 3. [c] BHT = 2,6-di-*tert*-butyl-4-methoxyphenol; standard control substance.

The antioxidative effect of pure compounds **1**−**11** on the autoxidation of linoleic acid was also determined. The values of AA measured at t = 60 and 120 min, employing bleaching of β-carotene as a model system, are reported in **Table 3**. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are the targets of lipid peroxidation. It is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activities. All tested compounds were weakly active in this test with respect to the reference compound BHT (*14*). The most active compounds were **10** and **11**, even though at t = 120 min their activities strongly decreased.

DISCUSSION

From our results on the on-line analysis of *S. birrea* methanol extracts it is evident that there is a close similarity of flavonoidic fractions of wild and cultivated plants. Our HPLC-UV/PDA and LC-MS chromatograms of both MeOH extracts revealed similar profiles (see **Figure 1**) with three predominant flavonoids (compounds **2**−**4**). The highest concentration of phenolic compounds was observed in the leaves of the wild plants on the basis of the total absorbance of the chromatograms at 254 nm that provide an approximated measure of their presence in the analyzed extracts. It seems that the MeOH extract of the wild plants is 3 times richer in flavonol compounds (see **Figure 3A**). Although the wild species has a higher amount of phenolic compounds, characteristic of the cultivated plant is the presence of galloylate catechins, well-known preventive antioxidants

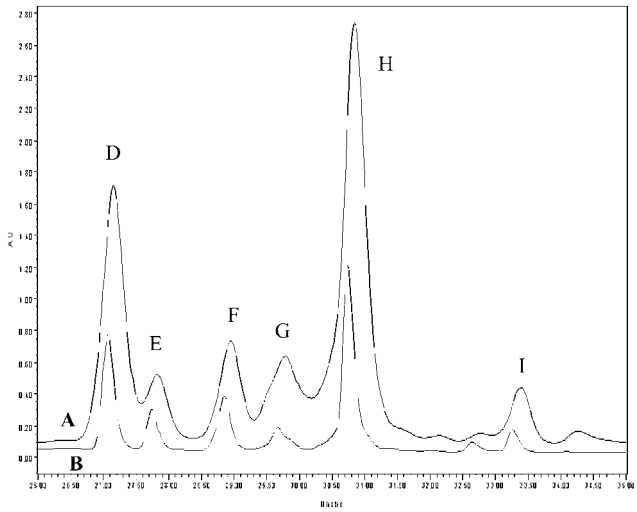

Figure 3. Quantitative HPLC comparison between flavonol glycosides of wild (A) and cultivated (B) *S. birrea* leaves monitored at 254 nm on the basis of their UV absorbance. (For chromatographic conditions see Materials and Methods.)

contained in green tea, that act as radical scavengers of reactive oxygen species.

In the TEAC assay, there appears to be a trend to increasing TEAC values with the number of free hydroxyls. Therefore, "adding" a gallic acid unit to a epicatechin molecule increases the hydrogen-donating ability both by increasing the number of free hydroxyls and by the introduction of a carbonyl function adjacent to a double bond by the ester linkage. This is the probably why the TEAC results of compounds **10** and **11** are higher than those of quercetin derivatives. In the literature it is also reported that the flavonol C-3 hydroxyl group is responsible for the high inhibition of β-carotene oxidation in the heterogeneous system (*19*). Comparison of the antioxidant activity of flavonol aglycons with the activity of their glycosides showed that the blockage of the C-3 hydroxyl group resulted in a total loss of antioxidant activity. Our AA results are in agreement with this general consideration, all compounds having lower activity than the synthetic antioxidant BHT.

There is a great deal of evidence indicating that excessive free radical production and lipid peroxidation are actively involved in the pathogenesis of a number of diseases including atherosclerosis (*20*), ischemia (*21*), carcinogenesis (*22*), neurodegenerative disorders (*23*), and diabetes (*24*, *25*). Supplementation of the natural endogenous antioxidant defense system through a balanced diet containing fruits, vegetables, or natural beverages derived from them could protect against various oxidative stresses. Nowadays, *S. birrea* plays an important role in the traditional medicine of Malian culture also as ITM, being used for the treatment of diabetes disorder and anti-inflammatory alteration. The established use of an *S. birrea* preparation ("diabetisane") for the treatment of diabetes might be attributed, to some degree, to the antioxidant activity of compounds such as flavonols and phenolic derivatives. In fact, a strict correlation has been reported between the hypoglycemic activity of herbal drugs and the presence of phenolic compounds that showed also capillary protection activity helpful for the prevention of degenerative diseases related to diabetes (*24*, *25*). The similar chemical compositions of methanol extracts of wild and cultivated *S. birrea* leaves and the presence of galloyl epicatechin derivatives in the cultivated species could permit the use of *S. birrea* cultivated plants instead of wild species in the preparation of "diabetisane".

LITERATURE CITED

(1) Iwu, M. M. In *Handbook of African Medicinal Plants*; CRC Press: Boca Raton, FL, 1993.

(2) Galvez, J.; Zarzuelo, A.; Crespo, M. E.; Utrilla, M. P.; Jimenez, J.; Spiessens, C.; De Witte, P. Antidiarrhoeic activity of *Sclerocarya birrea* bark extracts and its active tannin constituents in rats. *Phytother. Res.* **1991**, *5*, 276−278.

(3) Galvez, J.; Crespo, M. E.; Zarzuelo, A.; De Witte, P.; Spiessens, C. Pharmacological activity of a procyanidin isolated from *Sclerocarya birrea* bark: antidiarrhoeal activity and effect on siolated guinea-pig ileum. *Phytother. Res.* **1993**, *7*, 25−28.

(4) Galvez Peralta, J.; Zarzuelo, A.; Busson, R.; Cobbaert, C.; De Witte, P. (−)-epicatechin-3-galloylester: a secretagogue compound from the bark of *Sclerocarya birrea*. *Planta Med.* **1992**, *58*, 174−175.

(5) Belemtougri, R. G.; Constantin, B.; Cognard, C.; Raymond, G.; Sawadogo, L. Effects of *Sclerocarya birrea* (A. Rich.) Hochst (Anacardiaceae) leaf extracts on calcium signalling in cultured rat skeletal muscle cells. *J. Ethnopharmacol.* **2001**, *76*, 247−252.

(6) MSPAS. In *Formulaire Therapeutique National*; Ministhère de la Santé: Bamako, Mali, **1998**.

(7) Agrawal, P. K. In *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, The Netherlands, 1989.

(8) Collins, F. W.; Bohm, B. A.; Wilkins, C. K. Flavonol glycosides gallate from *Tellima grandiflora*. *Phytochemistry* **1975**, *14*, 1099−1102.

(9) Nicollier, G.; Thompson, A. C. Flavonoids of *Desmanthus illinoensis*. *J. Nat. Prod.* **1983**, *46*, 112−117.

(10) Sakar, M. K.; Kolodziej, H. Flavonoid glycosides from the flowers of *Prunus spinosa*. *Fitoterapia* **1993**, *64*, 180−181.

(11) Nawwar, M. A. M.; Buddrus, J.; Bauer, H. Dimeric phenolic constituents from the roots of *Tamarix nilotica*. *Phytochemistry* **1982**, *21*, 1755−1758.

(12) Miketova, P.; Schram, K. H.; Whitney, J. L.; Kerns, E. H.; Valcic, S.; Timmermann, B. N.; Volk, K. J. Mass spectrometry of selected components of biological interest in green tea extracts. *J. Nat. Prod.* **1998**, *61*, 461−467.

(13) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(14) Pratt, D. E. In *Phenolic Compounds in Food and their Effects on Health*; Huang, M. T., Lee, C. Y., Eds.; ACS Symposium Series 507; American Chemical Society: Washington, DC, 1992; Vol. II, pp 54−71.

(15) Wollenveber, E. Flavones and flavonols. In *The Flavonoids—Advances in Researches*; Harborne, J. B., Mabry, T. J., Eds.; Chapman and Hall: London, U.K., 1982; pp 240−242.

(16) Nakagawa, K.; Ninomiya, M.; Okubo, T.; Aoi, N.; Juneja, L. R.; Kim, M.; Yamanaka, K.; Miyazawa, T. Tea catechin supplementation increases antioxidant capacity and prevents phospholipid hydroperoxidation in plasma of humans. *J. Agric. Food Chem.* **1999**, *47*, 3967−3973.

(17) Agrawal, P. K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* **1992**, *23*, 3307−3330.

(18) Lien, E. J.; Ren, S.; Bui, H.-H.; Rubin, W. Quantitative structure activity relationship analysis of phenolic antioxidants. *Free Radical Biol. Med.* **1999**, *26*, 285−294.

(19) Mora, A.; Payà, M.; Rios, J. L.; Alcaraz, M. J. Structure−activity relationship of polymethoxy flavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation. *Biochem. Pharmacol.* **1990**, *40*, 793−797.

(20) Parthasarathy, S.; Santamam, N.; Ange, N. Oxidised low-density lipoprotein, a two-fased Janus in coronary artery disease. *Biochem. Pharmacol.* **1998**, *56*, 279−284.

(21) Keller, J. N.; Kindly, M. S.; Holtberg, F. W.; St. Clair, D. K.; Yen, H. C.; Germeyer, A.; Steiner, S. M.; Bruce-Keller, A. J.; Hutchins, J. B.; Mattson M. P. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite products, lipid peroxidation, and mitochondrial dysfuntion. *J. Neurosci*. **1998**, *18*, 687−697.

(22) Kamat, J. P.; Devasagayam, T. P. A. Oxidative damage to mitochondrial in normal and cancer tissues, and its modulation. *Toxicology* **2000**, *155*, 73−82.

(23) Perry, J.; Raine, K. A.; Nunomura, A.; Watayc, T.; Sayre, L. M.; Smith, M. A. How important is oxidative damage? Lessons from Alzheimer's disease. *Free Radical Biol. Med.* **2000**, *28*, 831−834.

(24) Spitaler, M. M.; Graier, W. F. Vascular targets of redox signaling in diabetes mellitus. *Diabetologia* **2002**, *45*, 475−494.

(25) Gorogawa, S.-I.; Kajimoto, Y.; Umayahara, J.; Kaneto, H.; Watada, H.; Kuroda, A.; Kawamori, D.; Yasuda, T.; Matsuhisa, M.; Yamasaki, Y.; Hori, M. Probucol preserves pancreatic-cell function through reduction of oxidative stress in type 2 diabetes. *Diabetes Res. Clin. Pract*. **2002**, *57*, 1−10.

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