Article

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Antioxidant Constituents of *Geranium bellum* Rose

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Abstract. Radical scavenging assay-guided fractionation of the antioxidant EtOAc and MeOH extracts from the aerial parts of *Geranium bellum* resulted in the isolation of β-sitosterol 3-*O*-β-D-glucopiranoside (**1**), quercetin 3-*O*-α-L-(2”-*O*-acetyl)arabinofuranoside (**2**), quercetin 3-*O*-α-L-arabinofuranoside (**3**), quercetin (**4**), methyl gallate (**5**), gallic acid (**6**), methyl brevifolin carboxylate (**7**), and dehydrochebulic acid trimethyl ester (**8**). These substances were fully characterized by 1D and 2D NMR spectroscopy as well as by their physical properties. 2,2’-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities for the extracts, primary fractions and pure compounds were determined. Compounds **2**-**4** showed very strong ABTS radical scavenging activity, whereas compound **5-8** had moderate activity and compound **1** was weakly active. Compounds **2**, **7** and **8** are isolated for the first time from *Geranium*.
Key words: *Geranium bellum* Rose, Geraniaceae, antioxidant activity, flavonoids, tannins, ^{1}H and ^{13}C NMR spectroscopy.

Resumen. La separación dirigida mediante captura de radicales libres de los extractos antioxidantes de AcOEt y MeOH de las partes aéreas de *Geranium bellum* condujo al aislamiento de 3-*O*-β-D-glucopiranósido de β-sitosterol (**1**), 3-*O*-α-L-(2”-*O*-acetil)arabinofuranósido de quercetina (**2**), 3-*O*-α-L-arabinofuranósido de quercetina (**3**), quercetina (**4**), galato de metilo (**5**), ácido gálico (**6**), brevifolin carboxilato de metilo (**7**) y el éster trimetílico del ácido dehidrochebúlico (**8**). Las sustancias fueron completamente caracterizadas mediante espectroscopía de RMN en 1D y 2D, así como por sus propiedades físicas. Se determinó las capacidades de captura de radicales del ácido 2,2’-azino-bis(3-etilbenzotiazolina-6-sulfónico) (ABTS) para los extractos, fracciones primarias y compuestos puros. Los compuestos **2**-**4** mostraron fuerte actividad de captura de radicales, mientras que los compuestos **5-8** presentaron actividad moderada y el compuesto **1** fue débilmente activo. Esta es la primera vez que los compuestos **2**, **7** y **8** se aíslan de *Geranium*.
Palabras clave: *Geranium bellum* Rose, Geraniaceae, actividad antioxidante, flavonoides, taninos, espectroscopía de RMN de ^{1}H y ^{13}C.

Introduction

The Geraniaceae family consists of some 11 genera with about 800 species widely distributed in temperate and subtropical regions of both hemispheres. Some species are used in traditional folk medicine while others, mainly those belonging to the *Pelargonium* (“geranium”, “malvón”) and *Geranium* genus, are cultivated for ornamental purposes and for the aromatic oils that they contain [1]. *Geranium* consists of *ca* 300 species, at least 13 of which are found in Mexico, mainly in coniferous forests, where their floral beauty makes them readily visible [1]. Some *Geranium* species have been employed in Mexican folk medicine as antifebrile, purgative, and antidiarrhetic agents, and for the relief of kidney pain [2-4]. Chemical examination of this genus has demonstrated that hydrolysable tannins, phenolic compounds and flavonoids are present in significant amounts [2-6]. The crystalline tannin geraniin [6] is claimed to be the main component in these plants. In recent times, A-type proanthocyanidins, designated geranins A, B, C and D, which showed significant antioxidant and antiprotozoal activity, were isolated from *Geranium niveum* [2, 3, 7].

Geranium bellum Rose is a perennial plant with long roots, found in the grassy meadows bordering pine/oak forests in the mountains of Hidalgo state, Mexico, where it has the popular name “pata de león” and has been used as a traditional remedy for treatment of fevers, pain, and gastrointestinal disorders [8]. This paper describes the radical scavenging assay-guided (antioxidant activity) fractionation of the EtOAc and MeOH extracts from the aerial parts of this species. β-Sitosterol 3-*O*-β-D-glucopyranoside (**1**) [9], quercetin 3-*O*-α-L-(2”-*O*-acetyl)-arabinofuranoside (**2**) [10], and quercetin 3-*O*-α-L-arabinofuranoside (**3**) (avicularin) [11] were found in the EtOAc extract, while the MeOH extract afforded compounds **1**-**3**, quercetin (**4**) [12], methyl gallate (**5**) [13], gallic acid (**6**) [13], methyl brevifolin carboxilate (**7**) [14], and dehydrochebulic acid trimethyl ester (**8**) [14] (Fig. 3). Antioxidant activity of these extracts (both initial fractions and pure compounds), was tested by measuring their capacity to scavenge 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals, an assay widely used for screening of antioxidant activity of natural products [15].

Results and Discussion

Extracts of the aerial parts showed high (EtOAc), moderate (MeOH) and low (H_2O) radical scavenging activity when analyzed by the ABTS free radical technique (Fig. 1). Chromatographic separation of EtOAc extract gave several initial fractions, which were tested for their ABTS free radical scavenging ability. Major activity was observed in fractions

C–K from which compounds **1**-**3** were isolated by successive chromatographic separations on silica gel.

Sequential diferential solubilization of the crude MeOH extract with acetone and EtOAc allowed gross separation of plant constituents. Thus, addition of acetone to the crude extract allowed separation of a non-soluble (A-NS) portion and a soluble (A-S) portion. This A-S fraction was concentrated in vacuo and to the resulting residue EtOAc was added giving both soluble (E-S) and non-soluble (E-NS) fractions. All these fractions were analyzed for their antioxidant activity. The EtOAc soluble (E-S) residue showed the best activity and was separated by CC to afford the active initial fractions M-2 to M-5 from which compounds **1**-**8** were obtained (Fig. 3). These substances were fully characterized by 1D and 2D NMR spectroscopy as well as by their physical properties. Compounds **2**-**4** showed very strong ABTS free radical scavenging activity, whereas compound **5**-**8** had moderate activity and compound **1** was only weakly active (Fig. 2). Compound **2**, **7** and **8** were isolated for the first time from *Geranium*, whereas substances **1** and **3**-**6** have been obtained from other *Geranium* species [2-5] or from the related genus *Pelargonium* [16].

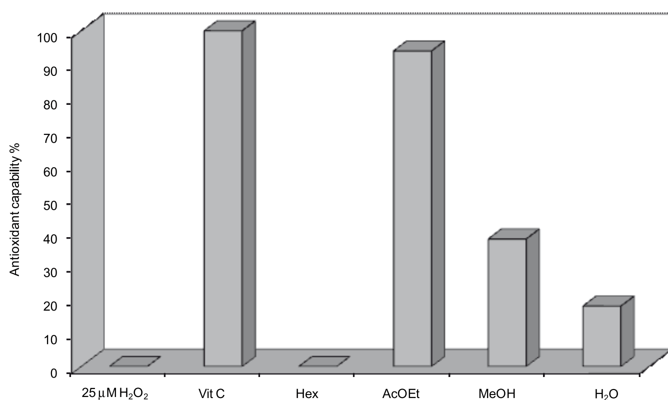

Fig. 1. Reduction of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation in the presence of extracts from *G. bellum*, and Vitamin C (ascorbic acid).

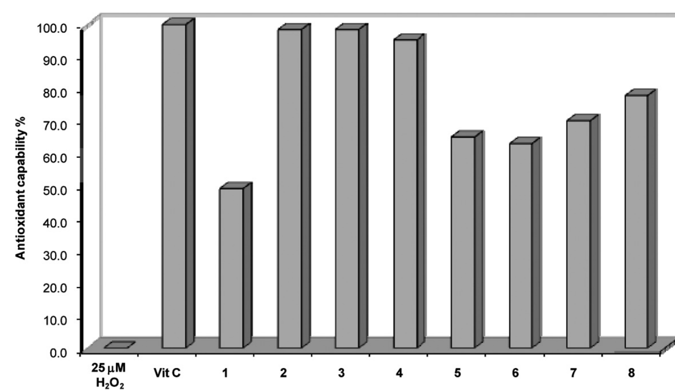

Fig. 2. ABTS radical scavenging capability of compounds **1**-**8** isolated from *G. bellum*, and Vitamin C (ascorbic acid).

Compound **2** was recently described as a metabolite from *Berchemia floribunda* (Wall.) Brongn [10] although optical rotation data were not reported. In addition, reported ^{1}H-NMR data for compound **2**, recorded in C_5D_5N [10], does not agree with our data measured in that solvent, but do agree with those determined in CD_3OD (Table 1). Our ^{13}C-NMR spectral data (in deuteropyridine) is in full agreement with that reported (Table 2). It should be noted that in the ^{1}H-NMR spectrum of compound 2, measured in C_5D_5N, the chemical shifts of H-8 and H-6 switch their relative positions with respect to spectra in other solvents (DMSO-d_6 or CD_3OD) (See Table 1). This observation was confirmed through detailed analysis of HMQC and HMBC spectra.

Methyl brevifolin carboxylate (**7**) and the trimethyl ester of dehydrochebullic acid (**8**) have been described as natural products isolated from *Phyllanthus urinaria* L. [14] and from *Jussiaea erecta* L. [17]. The absolute configuration at C-8 of compound **7** has never been described previously. A possible easy racemization of this labile proton was inferred [18]. Compound **7**, isolated in this work, did not show significant optical rotation. The other physical properties and the spectroscopic data corresponded well to those reported [14]. The physical constants and spectroscopic data for **8** obtained in this work are in good agreement with those reported [14, 17], except for the magnitude of the optical rotation which differs from that reported: $[\alpha]_D^{20}$ = + 169.3 (*c* 0.228, MeOH) (Lit. [14] $[\alpha]_D$ = + 28.5, MeOH). The *Z* geometry and (2*S*,3*S*) absolute configuration of compound **8** have been described and correlated with chebulagic acid [19], and are in agreement with the stereochemistry, determined by a NOESY experiment, of this natural substance isolated from *Jussiaea erecta* [17].

Experimental

General. IR spectra were measured as dispersions in KBr on a Perkin Elmer 2000 FT-IR spectrophotometer. Optical rotations were determined in MeOH on a Perkin Elmer 341 polarimeter. NMR measurements were performed at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Jeol Eclipse 400 spectrometer as CD_3OD, DMSO-d_6 or C_5D_5N solutions. Mass spectra were recorded at 70 eV on a Hewlett Packard 5890 Series II spectrometer. Column chromatography (CC) was carried out on Merck silica gel 60 (Aldrich, 230-400 mesh ASTM).

Plant Material. Specimens of *Geranium bellum* Rose were collected in the municipality of Epazoyucan, in Hidalgo State, México, during June, 2004. A voucher specimen (J. M. Torres Valencia 59) is preserved in the Herbarium of the Biological Research Center, Universidad Autónoma del Estado de Hidalgo, Mineral de la Reforma, Hidalgo, Mexico, and was identified by Professor Manuel González Ledesma of that institute.

Extraction and Isolation. The air-dried aerial parts of the plant (1.5 kg) were extracted successively with hexane, EtOAc, MeOH and H_2O. Extractions in organic solvents were each

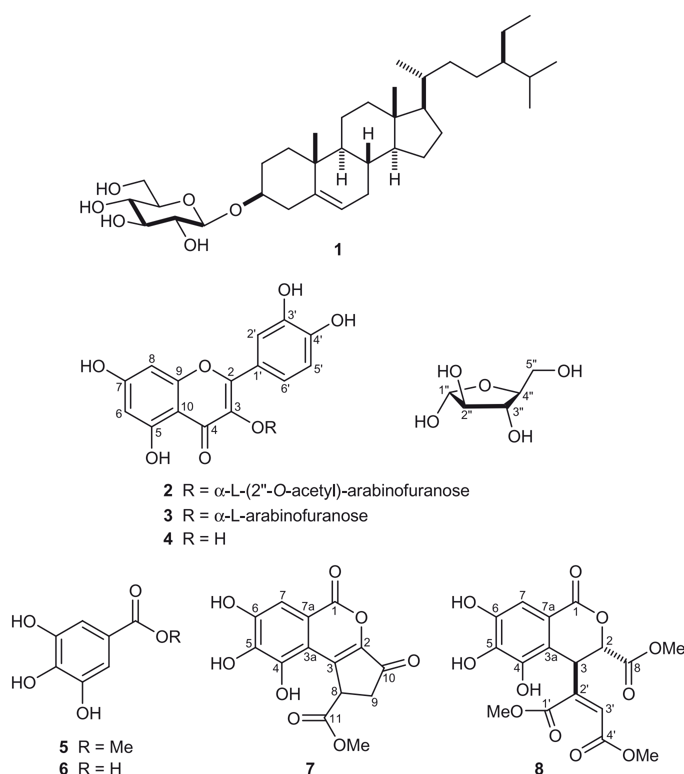

Fig. 3. The structure of the antioxidant compounds isolated from *G. bellum*.

conducted heating the solid plant residue in the appropriate solvent at reflux for 6 h, while the water extract was obtained by maceration at room temperature for 7 days. Filtration and evaporation of the extracts afforded green viscous oils (hexane, 8 g; EtOAc, 20 g; MeOH, 412 g and water, 120 g). Hexane and EtOAc extracts were dissolved in MeOH at 50 °C, then left at 0 °C for 12 h, insoluble fatty materials were finally removed by filtration. The filtrate was evaporated under vacuum to give defatted extracts. ABTS radical scavenging capacity of all extracts was examined and showed that EtOAc and MeOH extracts had the major activity. AcOEt extract (5 g) was chromatographed using silica gel (50 g) and eluting with a hexane-EtOAc gradient (9:1), (8:2), (7:3), (1:1), (1:4) and then with EtOAc, EtOAc-MeOH (9:1), (7:3), (1:1), MeOH and $(CH_3)_2CO$-H_2O (7:3). Fractions of 0.5 L of each polarity were collected to give fractions A to K, which were tested for their ABTS radical scavenging ability. The active fractions C–K (1.5 g) were combined and later separated by column chromatography (CC) with $CHCl_3$-MeOH-H_2O (25:7.5:1; 0.5 L), (11.5:7:1; 0.5 L) and (2:6:1; 0.5 L), collecting sixty 25 mL fractions. Further chromatography of the residue contained in fractions 7-13 using $CHCl_3$-MeOH (20:1) afforded compound **1** (35 mg). Sequential CC of residue contained in fractions 14-19 with $CHCl_3$-$(CH_3)_2CO$-H_2O (25:12:0.2) gave **2** (3 mg), whereas CC of the residue from fractions 20-34 using $CHCl_3$-$(CH_3)_2CO$-H_2O (20:12:0.2) provided **3** (2 mg).

Sequential differential solubilization of the crude MeOH extract (100 g) with acetone and EtOAc allowed gross separation of plant constituents. Thus, addition of acetone (3 × 500 mL) to the crude extract allowed separation of a non-soluble (A-NS) portion and a soluble (A-S) portion. This A-S fraction was concentrated in vacuo and to the resulting residue EtOAc (3 × 500 mL) was added giving both soluble (E-S) and non soluble (E-NS) fractions. All these fractions were analyzed for their antioxidant activity.

The E-S materia (10 g) was combined with 7 g of silica gel and chromatographed using silica gel (22 g) and $CHCl_3$ (0.1 L), $CHCl_3$-$(CH_3)_2CO$-H_2O (30:10:0.2; 0.1 L), $CHCl_3$-$(CH_3)_2CO$-H_2O (25:10:0.2; 0.1 L), $CHCl_3$-$(CH_3)_2CO$-H_2O (15:10:0.2; 0.1 L), $CHCl_3$-$(CH_3)_2CO$-H_2O (10:10:0.2; 0.1 L), EtOAc-MeOH-H_2O (10:10:0.2; 0.15 L). Fractions of each polarity were obtained and marked as M1-M6. These fractions were tested for their ABTS radical scavenging activ-

Table 1. ^{1}H (400 MHz) NMR chemical shifts and coupling constants of compound **2**.

	δ_H/ppm (mult., *J* in Hz)		
Proton	CD_3OD[a]	DMSO-d_6[b]	C_5D_5N[c]
6	6.19 (d, 2.2)	6.20 (d, 2.2)	6.71 (d, 2.2)
8	6.37 (d, 2.2)	6.42 (d, 2.2)	6.66 (d, 2.2)
2’	7.50 (d, 2.2)	7.46 (d, 2.2)	8.25 (d, 2.2)
5’	6.89 (d, 8.0)	6.85 (d, 8.5)	7.38 (d, 8.4)
6’	7.48 (dd, 8.0, 2.2)	7.52 (dd, 8.5, 2.2)	7.99 (dd, 8.4, 2.2)
1”	5.63 (br s)	5.71 (br s)	6.56 (br s)
2”	5.34 (dd, 3.3, 0.7)	5.25 (dd, 3.6, 1.0)	6.11 (dd, 3.0, 0.7)
3”	4.04 (dd, 6.2, 3.3)	3.93 (dd, 7.0, 3.6)	4.88 (dd, 6.5, 3.0)
4”	3.86 (ddd, 6.2, 4.7, 3.7)	3.62 (ddd, 7.0, 4.4, 3.3)	4.73 (ddd, 6.5, 4.7, 3.3)
5”a	3.53 (d d, 12.5, 3.7)	3.35 (dd, 12.0, 3.3)	4.11 (dd, 12.0, 3.3)
5”b	3.48 (dd, 12.5, 4.7)	3.29 (dd, 12.0, 4.4)	4.05 (dd, 12.0, 4.7)
OCO$\underline{C}H_3$	2.09 (s)	2.08 (s)	1.94 (s)

[a]CH_3 solvent residual peak referenced at 3.31 ppm. [b]Solvent residual peak referenced at 2.50 ppm. [c]H-2 and H-6 solvent residual peak referenced at 8.74 ppm.

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Table 2. ^{13}C (100 MHz) NMR chemical shifts of compound **2**.

Carbon	δ_C/ppm		
	CD_3OD[a]	DMSO-d_6[b]	C_5D_5N[c]
2	157.9	156.9	157.9
3	133.4	133.0	134.1
4	178.3	177.5	178.7
5	161.7	161.2	162.7
6	98.5	98.7	99.6
7	164.6	164.3	165.7
8	93.4	93.4	94.4
9	157.1	156.4	157.5
10	104.4	105.1	105.1
1’	121.6	120.7	122.0
2’	115.5	115.6	117.0
3’	154.0	145.2	147.0
4’	148.5	148.6	150.6
5’	115.1	115.6	116.5
6’	121.8	121.7	122.3
1”	105.7	103.9	106.4
2”	84.3	84.0	85.7
3”	75.4	74.6	76.3
4”	86.0	85.5	87.5
5”	60.6	59.8	61.5
$O\underline{C}OCH_3$	170.4	169.6	170.0
$OCO\underline{C}H_3$	19.4	20.7	20.5

[a]Solvent peak referenced at 48.0 ppm. [b]Solvent peak referenced at 39.5 ppm. [c]C-2 and C-6 solvent peak referenced at 150.0 ppm.

ity. Fractions M2 to M5 showed the major activity. Column chromatography of fraction M2 (258 mg) with $CHCl_3$ (6 mL), $CHCl_3$-MeOH (10:0.1; 44 mL), $CHCl_3$-MeOH (10:0.15; 40 mL) and $CHCl_3$-MeOH (10:0.2; 10 mL), collecting eluates of 2 mL, gave fractions M2-1 (eluates 1-13), M2-2 (14-34) and M2-3 (35-53).

CC of Fraction M3 (1.033g) using $CHCl_3$ (20 mL), $CHCl_3$-MeOH (50:0.5; 20 mL), $CHCl_3$-MeOH (50:0.75; 48 mL), $CHCl_3$-MeOH (50:1; 42 mL), $CHCl_3$-MeOH (50:1.25; 36 mL), $CHCl_3$-MeOH (50:1.5; 36 mL), $CHCl_3$-MeOH (50:2; 40 mL), $CHCl_3$-MeOH (50:2.5; 40 mL), $CHCl_3$-MeOH (50:3; 40 mL), $CHCl_3$-MeOH (50:5 46 mL). Eluates of 2 mL were collected to give fractions M3-1 (eluates 1-25), M3-2 (26-61), M3-3 **(**62-106), M3-4 (107-140), M3-5 **(**141-173) and M3-6 **(**174-195).

From this fractions M2-2 (108 mg) and M3-2 (223 mg) were combined and chromatographed using $CHCl_3$ (24 mL), $CHCl_3$-$(CH_3)_2CO$ (50:0.3; 20 mL), $CHCl_3$-$(CH_3)_2CO$ (50:0.6; 20 mL), $CHCl_3$-$(CH_3)_2CO$ (50:1; 26 mL), $CHCl_3$-$(CH_3)_2CO$ (50:1; 26 mL) collecting eluates of 2 mL. From fractions 13-20 compound **8** (20 mg) was isolated, whereas fractions 54-67 afforded **5** (97 mg).

Fractions M2-3 (70 mg) and M3-3 (170 mg) were combined and purified by CC with $CHCl_3$ (28 mL), $CHCl_3$-$(CH_3)_2CO$ (50:0.5; 32 mL), $CHCl_3$-MeOH (50:1; 20 mL), $CHCl_3$-MeOH (50:2.5; 80 mL), and eluates of 2 mL were collected. Compound **1** was isolated from fractions 38-41 (6 mg), and fractions 52-79 gave **4** (60 mg).

CC of fraction M3-4 (122 mg), using $CHCl_3$ (28 mL), $CHCl_3$-MeOH (50:0.5; 20 mL), $CHCl_3$-MeOH (50:0.7; 60 mL), 92-115 $CHCl_3$-MeOH (50:1.2; 56 mL) and collecting eluates of 2 mL, afforded **6** (5 mg) from fractions 94-106. Fractions 48-93 (57 mg) were mixed and further chromatographed on silica gel with $CHCl_3$ (6 mL) and $CHCl_3$-MeOH (10:0.4; 64 mL) to give **2** (32 mg).

Fraction M3-5 (135 mg) was purified by CC with $CHCl_3$ (4 mL), $CHCl_3$-$(CH_3)_2CO$-H_2O (20:10:0.2; 48 mL), $CHCl_3$-$(CH_3)_2CO$-H_2O (19:10:0.2; 20 mL) and $CHCl_3$-$(CH_3)_2CO$-H_2O (15:10:0.2; 38 mL), and collecting eluates of 2 mL. Fractions 26-52 (38 mg) were combined and purified by CC using $CHCl_3$ (6 mL) and $CHCl_3$-MeOH-H_2O (20:3:0.2; 60 mL) to give **3** (22.5 mg).

Addition of MeOH to fraction M3-6 caused precipitation of a solid, which was crystallized from $(CH_3)_2CO$-H_2O (9:1) to give **7** (10 mg).

Quercetin 3-*O*-α-L-(2”-*O*-acetyl)arabinofuranoside (**2**). Yellow amorphous powder; mp 190–192 °C; $[\alpha]_D^{20}$ – 91.4 (*c* 0.56, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.23); 257 (2.97); 354 (2.89); nm; IR (KBr disk) ν_{max} 3441, 3308, 2900, 1732, 1652, 1603, 1573, 1453, 1345, 1305, 1259, 1201, 1161, 998, 947 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1 and Table 2, respectively.

Methyl brevifolin carboxylate (**7**). Pale yellow amorphous powder; mp 256-259 °C (crystallized from acetone); IR (KBr disk) ν_{max} 3417, 3100, 2924, 1695, 1630 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.29 (1H, s, H-7), 4.42 (1H, dd; *J* = 7.7, 1.8 Hz, H-8), 3.62 (3H, s, OMe), 2.98 (1H, dd, *J* = 18.7, 7.7 Hz, H-9a), 2.46 (1H, dd, *J* = 18.7, 1.8Hz, H-9b); ^{13}C NMR (DMSO-d_6, 100 MHz, assignments by APT and HMQC) δ 193.0 (C-10), 172.5 (C-11), 160.1 (C-1), 149.6 (C-5), 145.8 (C-2), 143.5 (C-4), 140.3 (C-6), 138.4 (C-3), 114.9 (C-3a), 112.9 (C-7a), 108.1 (C-7), 52.0 (OMe), 40.6 (C-8), 36.9 (C-9).

Dehydrochebulic acid trimethyl ester (**8**). White amorphous powder; mp 200–203 ºC (decomp.); $[\alpha]_D^{20}$ + 169.3 (*c* 0.228, MeOH); IR (KBr disk) ν_{max} 3397, 3257, 2959, 2923, 1759, 1708, 1618 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz, H-2 and H-6 solvent residual peak referenced at 8.74 ppm) δ 7.93 (1H, s, H-7), 7.13 (1H, s, H-3’), 6.20 (1H, br s, H-3), 5.89 (1H, d, *J* = 1.4 Hz, H-2), 3.68 (3H, s, OMe), 3.54 (6H, s, 2 × OMe); ^{13}C NMR (C_5D_5N, 100 MHz, C-2 and C-6 solvent peak referenced at 150.0 ppm) δ 170.5 (C-8), 166.7 (C-1’), 165. 7 (C-4’), 164.6 (C-1), 147.4 (C-6), 145.0 (C-4), 142.8 (C-2’), 141.2 (C-5), 129.6 (C-3’), 117.5 (C-7a), 116.1 (C-3a), 108.6 (C-7), 79.2 (C-2), 52.7 (OMe), 52.2 (OMe), 51.9 (OMe), 35.3 (C-3).

Radical-scavenging assays. Radical scavenging activities of fractions of EtOAc extract, and of pure isolated compounds were determined by using the $ABTS^{\bullet+}$ assay method [15]. A Stock solution of ABTS 10 mM in phosphate-buffer 0.1 M pH

6 was prepared and allowed to stand at room temperature in the dark. The $ABTS^{•+}$ solution was diluted to 1 mM in phosphate-buffer. The reaction mixture consisted of 500 μL $ABTS^{•+}$ (1 mM solution), 25 μM of H_2O_2, 10 μL of horseradish peroxidase at 500 μg/mL and 1410 μL of phosphate-buffer, the amount of extract was 1.5 mg/mL. Vitamin C was used as reference substance at the same concentration as the extract and compounds.

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