Full Paper

Flavanol Derivatives from *Rhizophora stylosa* and Their DPPH Radical Scavenging Activity

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Abstract: A new acetylated flavanol, 3,7-*O*-diacetyl (–)-epicatechin (**3**), and seven known flavanol derivatives, (–)-epicatechin (**1**), 3-*O*-acetyl (–)-epicatechin (**2**), 3,3′,4′,5,7-*O*-pentaacetyl (–)-epicatechin (**4**), (+)-afzelechin (**5**), (+)-catechin (**6**), cinchonain Ib (**7**), and proanthocyanidin B2 (**8**), were isolated from the stems and twigs of the mangrove plant *Rhizophora stylosa* and identified. The crude extract, the different fractions and all of the purified compounds were evaluated for DPPH radical scavenging activity.

Keywords: Rhizophoraceae, *Rhizophora stylosa*, flavanol, radical scavenging activity

Introduction

Mangrove plants are well-known as a rich source of tannins. The bark of true mangrove plants contains about 1%~30% tannins [1]. The vegetable tannins or plant polyphenols group is comprised of two classes of compounds, the "hydrolysable" and the "condensed" tannins. Flavan-3-ols constitute the latter group [2]. Some species of the mangrove plant genus *Rhizophora* are used by the local people as folk medicines for many diseases. For example, the bark of *R. mucronata* is used for the
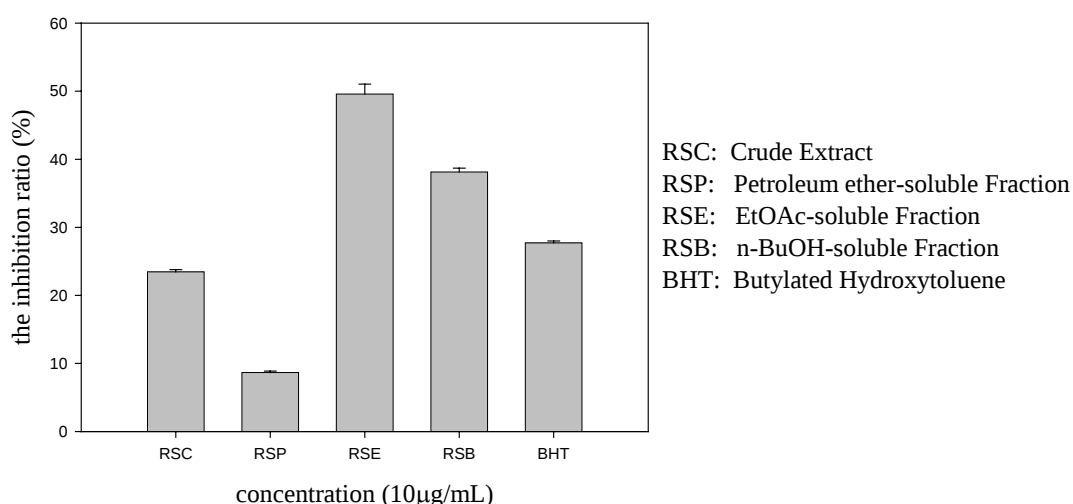
treatment of diabetes in India [3], and when boiled in water it is used as an astringent for diarrhea, nausea, and vomiting and as an antiseptic in Thailand [4]. The extract of some species of this genus has been reported to have antifungal activity [5], antibacterial activity [6], anti-inflammatory activity [7], gastric antiulcer properties [8], efficacy in wound healing [9], and a protective role in naphthalene-induced mitochondrial dysfunction [10]. Furthermore, the major active principles of these pharmacological and biomedicinal properties are polyphenols that behave as antioxidants.

During our phytochemical investigation of *Rhizophora stylosa* Griff (Rhizophoraceae), one new acetylated flavanol, 3,7-*O*-diacetyl (–)-epicatechin (**3**), and seven known flavanol derivatives **1**, **2**, **4**–**8** were isolated from the stems and twigs of this plant and identified. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the crude extract, the different fractions, and all of the purified compounds were evaluated.

Results and Discussion

The antioxidant activity of the crude extract (RSC), the petroleum ether-soluble fraction (RSP), the EtOAc-soluble fraction (RSE), and the *n*-BuOH-soluble fraction (RSB) were determined using the DPPH radical assay, as described in our previous report [11]. As shown in Figure 1, RSE and RSB were demonstrated to be the most active fractions. Further phytochemical investigation of these two fractions resulted in the isolation and characterization of compounds **1**–**8**. Each of these compounds displayed various levels of DPPH radical scavenging activity.
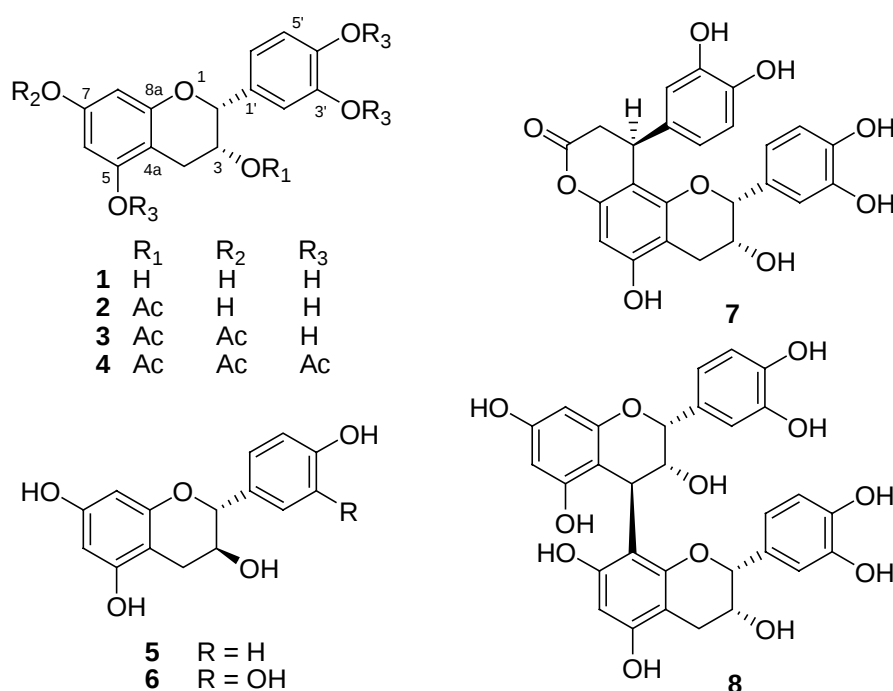
Figure 1. The inhibition ratio (percentage) of extract and different fractions derived from *R. stylosa* (at 10 μg/mL) in DPPH radical scavenging assay. The results are given as means ± SD (*n* = 3).

RSC: Crude Extract
RSP: Petroleum ether-soluble Fraction
RSE: EtOAc-soluble Fraction
RSB: n-BuOH-soluble Fraction
BHT: Butylated Hydroxytoluene

Compound **3** was obtained as a yellow oil and its molecular formula was determined to be $C_{19}H_{18}O_8$ by HR-ESI-MS ([M + Na]$^+$ *m/z* 397.0893, calcd. for $C_{19}H_{18}O_8Na$, 397.0899) which was in agreement with the ^1H- and ^{13}C-NMR spectral data. Its IR spectrum exhibited absorption bands at 3362 cm^{-1} for hydroxyl, at 1738 cm^{-1} for ester carbonyl, and at 1604 cm^{-1}, 1519 cm^{-1} and 1443 cm^{-1} for the phenyl functionalities in the molecule. The ^1H-NMR spectrum of **3** was characteristic of a flavanol,

revealing the presence of five aromatic protons at δ 7.01 (1H, br s, H-2′), 6.81 (2H, br s, H-5′ and H-6′), 6.32 (1H, d, J = 2.4 Hz, H-8) and 6.25 (1H, d, J = 2.4 Hz, H-6); two oxymethine protons at δ 5.37 (1H, m, H-3) and 5.08 (1H, d, J = 6.2 Hz, H-2) and two methylene group protons at δ 2.97 (1H, d, J = 4.7, 17.4 Hz, H-4a) and 2.79 (1H, d, J = 2.2, 17.4 Hz, H-4b). Detailed inspection of the NMR spectral data revealed that the chemical structure of **3** was very similar to that of (–)-epicatechin (**1**) [12], the difference being the presence of two methyl signals at δ 2.25 (3H, s, OCOC$\underline{H}_3$) and 1.86 (3H, s, OCOC$\underline{H}_3$), which showed direct correlation with carbonyl carbon signals at δ 169.0 and 170.3, respectively, in the HMBC spectrum. These signals clearly indicated the presence of two acetoxyl groups in **3**. The proton at δ 5.37 (H-3) showed a long-range correlation with the carbonyl carbon signal at δ 170.3 in the HMBC spectrum, indicating that one of the acetoxyl groups was attached to C-3. The second acetoxyl group was deduced to be attached at C-7, rather than at C-5, based on the fact that the ^{13}C-NMR signal of C-7 was upfield shifted (to δ 151.6) in **3,** compared to that of (–)-epicatechin (**1**) (δ 156.9). On the other hand, the chemical shift for the methylene carbon C-4 at δ 26.5 in **3** was similar to that of 3-*O*-acetyl (–)-epicatechin (**2**) (δ 26.4), indicating that the 5-OH group was not acetylated, otherwise, this signal would shift to a remarkably upfield position, i.e., δ 23.9, for a C-5 acetoxylated compound such as 3,3′,4′,5,7-*O*-pentaacetyl (–)-epicatechin (**4**). From the above evidence, the structure of compound **3** was determined to be 3,7-*O*-diacetyl (–)-epicatechin.

Figure 2. The chemical structure of compounds **1**-**8**.

	R₁	R₂	R₃
1	H	H	H
2	Ac	H	H
3	Ac	Ac	H
4	Ac	Ac	Ac

5	R = H
6	R = OH

The known compounds **1**, **2**, **4**–**8** were characterized as (–)-epicatechin (**1**) [12], 3-*O*-acetyl (–)-epicatechin (**2**) [13], 3,3′,4′,5,7-*O*-pentaacetyl (–)-epicatechin (**4**) [14], (+)-afzelechin (**5**) [15], (+)-catechin (**6**) [12], cinchonain Ib (**7**) [16], and proanthocyanidin B2 (**8**) [12], respectively, based on the detailed comparison of the ^{1}H- and ^{13}C-NMR spectral data with those of the literature reports. To the best of our knowledge, this is the first report of the isolation of compound **2** as a natural substance, as previously **2** had been obtained after hydrolysis of compound **4**. This strategy was used to protect

phenolic hydroxyl groups in modifications and synthesis of various bioactive natural products [13,17]. The ^{13}C-NMR spectral data of compounds **2** and **4** are reported here for the first time (see Experimental).

DPPH Radical Scavenging Activity of isolated compounds

The DPPH radical scavenging activity of compounds **1**-**8** was presented in Table.1.

Table 1. The IC_{50} (µg/mL) of compounds **1**–**8** in DPPH radical scavenging assay.

Compound	Chemical name	IC_{50} (µg/mL)
1 and **6**	(–)-epicatechin and (+)-catechin	6.5
2	3-*O*-acetyl (–)-epicatechin	15.0
3	3,7-*O*-diacetyl (–)-epicatechin	25.4
4	3,3′,4′,5,7-*O*-pentaacetyl (–)-epicatechin	>1000
5	(+)-afzelechin	36.7
7	cinchonain Ib	7.8
8	proanthocyanidin B2	4.3
BHT	butylated hydroxytoluene	18.0

The results revealed that except for compound **4**, compounds **1**–**8** isolated from *R. stylosa* displayed DPPH radical scavenging activity comparable to that of the positive control butylated hydroxytoluene (BHT). Compound **8** showed the strongest activity, with IC_{50} 4.3 µg/mL, four times more active than the positive control, BHT (IC_{50} 18.0 µg/mL). The DPPH radical scavenging activities of these compounds seemed to be related to the number of phenol groups. Data analysis revealed that compounds having adjacent hydroxyl groups on the ring-B possessed higher activity, a fact confirmed by the literature [18]. From the above data, it can be deduced that the main components responsible for the antioxidant activities of *R. stylosa* extracts were the flavanol derivatives. Further work is in progress to assess whether this species could be used as a source of natural antioxidant for pharmaceutical and food applications.

Experimental

General

Optical rotations were obtained on JASCO P-1020 digital polarimeter. UV spectra were recorded on *a* PuXi TU-1810 UV-visible spectrophotometer. IR spectra were taken on a Nicolet 510P FT-IR spectrometer. NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer using TMS as internal standard and chemical shifts were recorded as δ values (500 MHz for ^{1}H and 125 MHz for ^{13}C). ESI-MS were measured on a VG AutoSpec 3000 mass spectrometer. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China) and reversed-phase silica gel C$_{18}$ (40-75 µm, Fuji Silysia Chemical Ltd) were used for open column chromatography (CC). Sephadex LH-20

(18-110 μm) was obtained from Pharmacia. Thin-layer chromatography (TLC) was performed on precoated 0.2 mm thick silica gel 60 GF$_{254}$ plates (Qingdao Haiyang Chemical Factory, Qingdao, China); spots were detected by spraying with anisaldehyde–H$_2$SO$_4$ reagent, followed by heating.

Plant Material

The stems and twigs of *Rhizophora stylosa* Griff. were collected on Hainan Island on the South China coastline in July 2004 and authenticated by Prof. H. Peng at Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (0407A) was deposited at the Key Laboratory of Experimental Marine Biology of the Institute of Oceanology, Chinese Academy of Sciences.

Extraction and Isolation

The pulverized stems and twigs of *R. stylosa* (6.1 kg) were macerated three times with a mixture of CHCl$_3$/MeOH (1:1) at room temperature for 5 days for each time. The combined extracts were concentrated and yielded 827 g of a crude extract (RSC). This extract was suspended in water and then was further extracted with petroleum ether, EtOAc, and *n*-BuOH to yield 41 g of petroleum ether-soluble fraction (RSP), 109 g of EtOAc-soluble fraction (RSE), and 244 g of *n*-BuOH-soluble fraction (RSB), respectively. The RSE fraction was subjected to a column chromatography (CC) over silica gel eluted with different solvents of increasing polarity (petroleum ether/acetone, 3:1 and 1:1; then CHCl$_3$/MeOH, from 10:1 to 1:1) to yield 10 fractions (E-1~E-10) on the basis of TLC analysis. Fraction E-6 (1.0 g) was subjected to CC using petroleum ether-acetone (2:1) as an eluent to afford seven subfractions (E-6-1~E-6-7). E-6-6 (87.5 mg) was subjected to CC on Sephadex LH-20 using CHCl$_3$/MeOH (1:1) as an eluent to afford compound **5** (34.3 mg). E-7 (6.8 g) was subjected to CC on silica gel using CHCl$_3$/MeOH (12:1) as an eluent to afford four subfractions (E-7-1~E-7-9). E-7-7 (387.6 mg) was subjected to CC on Sephadex LH-20 using CHCl$_3$/MeOH (1:1) as an eluent to afford four subfractions (E-7-7-1~E-7-7-4). E-7-7-2 (67.9 mg) was subjected to CC on silica gel using petroleum ether/acetone (4:1) as an eluent to afford compounds **2** (6.2 mg), **3** (3.2 mg), and **4** (25.7 mg). E-8 (9.6 g) was subjected to VLC on silica gel using CHCl$_3$/MeOH (18:1, 10:1, 5:1, 4:1) as an eluent to afford four subfractions (E-8-1~E-8-4). E-8-3 was subjected to CC on Sephadex LH-20 using CHCl$_3$/MeOH (1:1) as an eluent, followed by reversed-phase silica gel C$_{18}$ chromatography using MeOH as an eluent to afford a mixture of compounds **1** and **6** (81.6 mg) and **7** (15.3 mg). The *n*-BuOH-soluble fraction was subjected to a column chromatography over silica gel eluted with CHCl$_3$/MeOH (5:1). The eluents were combined into 10 fractions (B-1–B-10). Fraction B-4 was subjected to Sephadex LH-20 chromatography using MeOH as an eluent to yield compound **8** (140.0 mg).

3-O-acetyl (–)-epicatechin (**2**): ^{13}C-NMR (acetone-d_6) δ: 170.3 (O$\underline{C}$OCH$_3$), 157.8 (C-7), 157.4 (C-8a), 157.0 (C-5), 145.6 (C-3′), 145.6 (C-4′), 131.3 (C-1′), 119.2 (C-6′), 115.6 (C-5′), 114.8 (C-2′), 98.8 (C-4a), 96.4 (C-6), 95.8 (C-8), 77.7 (C-2), 69.0 (C-3), 26.4 (C-4), 20.9 (OCO$\underline{C}$H$_3$).

3,7-O-diacetyl (–)-epicatechin (**3**): $[\alpha]_D^{25}$: – 6.85° (c 0.045, acetone); IR (KBr) cm^{-1}: 3,362, 2,925, 1,738, 1,626, 1,604, 1,519, 1,443, 1,372, 1,234, 1,046; UV λ_{max} (acetone) nm (log ε): 212 (2.79), 327 (2.20); ^{1}H-NMR (acetone-d_6) δ 7.01 (1H, br s, H-2′), 6.81 (2H, br s, H-5′ and H-6′), 6.32 (1H, d, J = 2.4 Hz, H-8), 6.25 (1H, d, J = 2.4 Hz, H-6), 5.37 (1H, m, H-3), 5.08 (1H, d, J = 6.2 Hz, H-2), 2.97 (1H, d, J = 4.7, 17.4 Hz, H-4a), 2.79 (1H, d, J = 2.2, 17.4 Hz, H-4b), 2.25 (3H, s, OCOC$\underline{H}_3$), and 1.86 (3H, s, OCOC$\underline{H}_3$); ^{13}C-NMR (acetone-d_6) δ: 170.3 (O$\underline{C}$OCH$_3$), 169.0 (O$\underline{C}$OCH$_3$), 157.7 (C-5), 156.9 (C-8a), 151.6 (C-7), 145.8 (C-3′),145.7 (C-4′), 130.8 (C-1′), 119.2 (C-6′), 115.7 (C-5′), 114.9 (C-2′), 104.6 (C-4a), 103.6 (C-8), 101.7 (C-6), 78.1 (C-2), 68.3 (C-3), 26.5 (C-4), 20.9 (OCO$\underline{C}$H$_3$), 20.8 (OCO$\underline{C}$H$_3$); ESI-MS: 413 [M + K]$^+$, 397 [M + Na]$^+$, 375 [M + H]$^+$, 360, 316, 301, 274, 156, 141,116; HR-ESI-MS: m/z 397.0893 (calcd for C$_{19}$H$_{18}$O$_8$Na, [M + Na]$^+$, 397.0899).

3,3′,4′,5,7-O-pentaacetyl (–)-epicatechin (**4**): ^{13}C-NMR (CDCl$_3$) δ: 170.1, 169.0, 168.3, 168.0, 168.0 (O$\underline{C}$OCH$_3$), 154.4 (C-8a), 149.8 (C-7), 149.4 (C-5), 142.1, 142.1 (C-3′, 4′), 136.1 (C-1′), 124.4 (C-6′), 123.7 (C-5′), 121.7 (C-2′), 110.2 (C-4a), 108.7 (C-6), 107.7 (C-8), 77.6 (C-2), 68.3 (C-3), 23.9 (C-4), 21.1, 20.9, 20.7, 20.6, 20.6 (OCO$\underline{C}$H$_3$).

DPPH Radical Scavenging Activity

The scavenging effects of samples on DPPH radicals were determined according to the method of literature [11]. Synthetic antioxidant BHT was used as positive control.

Acknowledgements

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Sample Availability: Samples of the compounds **1**–**8** are available from the authors.