Antioxidant compounds from ethanol extracts of bamboo (Neosinocalamus affinis) leaves

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Activity-guided fractionation of Neosinocalamus affinis leaves led to obtain two new flavonoids, 4′-O-((7\(^R\), 8\(^S\), S)-8\(^R\)-guaacylglycerl)-pleioside B (9) and apigenin 6-C-\(\beta\)-D-fucopyranosyl-7-\(\beta\)-D-glucopyranoside (10) along with eight known compounds. Their structures were elucidated on the basis of spectroscopic data (UV, IR, NMR, and MS). Among these 10 compounds, farobin A (4) and isoorientin (7) showed significant antioxidant activity evaluated by 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), superoxide anion and nitric oxide (NO) radical-scavenging assays.

Keywords: Neosinocalamus affinis; radical-scavenging effect; DPPH; ABTS; superoxide anion; nitric oxide

1. Introduction

Free radicals, a reactive chemical species with a single unpaired electron in an outer orbit, are generally formed in human body, which showed their beneficial effects on the organisms as signaling and regulatory molecules at physiological levels [1]. However, excess of free radicals could induce oxidative stress by causing oxidative damage to biological molecules such as proteins, lipids, carbohydrates, and nucleic acids, which was implicated in aging and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders, and other chronic conditions [2]. Antioxidants were deemed to any molecule capable of stabilizing or deactivating free radicals, and proved to prevent or treat many human diseases related to free radicals, which greatly stimulated interest in exploration and development of antioxidants, especially from plants [3].

Bamboo leaves were considered as a natural resource of antioxidants [4] without toxicity [5]. Nevertheless, a few investigations were carried out on the antioxidant activity and corresponding secondary metabolites of leaves of Neosinocalamus affinis [6], which was one of the most widely distributed species in Sichuan Province of China. Thus, a study on antioxidant effect and corresponding active components was carried out in order to take good use of N. affinis leaves. Ten compounds (1–10; Figure 1) were obtained based on activity-guided isolation, most of which showed scavenging effects on free radicals including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide anion, and NO.

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2. Results and discussion

Compound 9 was obtained as a yellow powder. Its molecular formula C$_{34}$H$_{38}$O$_{16}$ was provided by its HR-ESI-MS ($m/z$ 703.2244 [M + H]$^+$; 725.2074 [M + Na]$^+$). The UV spectrum showed the characteristic absorption at 249 and 329 nm for the presence of flavonoid skeleton. The IR spectrum showed absorption bands at 3436 (OH), 1634 (conjugated CO), 1600, 1496, and 1456 (aromatic C=C) cm$^{-1}$. In addition to the coupling constant of anomeric proton ($J = 7.6$ Hz), the detection of glucose in acid hydrolysis products of compound 9 on TLC (EtOAc–MeOH–H$_2$O, 15:6:2, v/v/v) indicated the presence of glucopyranosyl unit with $\beta$-D or $\alpha$-L configuration in compound 9.

By comparison of $^1$H and $^{13}$C NMR spectra of compound 9 (Table 1) with those of pleioside B (compound 1), additional signals for the gluaiacylglyceryl [1-(4-hydroxy-3-methoxyphenyl) glyceryl] group [7] were observed: $\delta_H$ 6.93 (1H, br s, H-2$''$), 6.75 (1H, br d, $J = 8.2$ Hz, H-6$''$), 6.70 (1H, d, $J = 8.0$ Hz, H-5$''$), 4.80 (1H, overlap, H-7$''$), 4.35 (1H, dd, $J = 8.9$, 5.0 Hz, H-8$''$), 3.74 (overlap, H-9$''a$), 3.50 (overlap, H-9$''b$); $\delta_C$ 133.2 (C-1$''$), 111.0 (C-2$''$), 147.0 (C-3$''$), 145.4 (C-4$''$), 114.7 (C-5$''$), 119.4 (C-6$''$), 72.2 (C-7$''$), 86.4 (C-8$''$), 60.2 (C-9$''$). Corresponding downfield shifts of related carbon signals for C-1$'$ ($\Delta\delta_C = 4.9$) and C-3$'$/C-5$'$ ($\Delta\delta_C = 4.8$) indicated that compound 9 may be 4'-O-gluaiacylglyceryl pleioside B, which was further confirmed by the key HMBC correlation (Figure 2) from H-8$''$ to C-4$''$.

The location of the methoxyl group [$\delta_H$ 3.92 (3H, s); $\delta_C$ 56.2] at C-7 (HMBC correlation from $\delta_H$ 3.92 to $\delta_C$ 163.7) and a glucopyranosyl moiety [$\delta_H$ 4.79 (1H, d, $J = 7.6$ Hz, H-1$''$); $\delta_C$ 104.0 (C-1$''$), 73.6 (C-2$''$), 75.8 (C-3$''$), 70.0 (C-4$''$), 77.6 (C-5$''$), 61.0 (C-6$''$)] at C-5 (HMBC correlation from $\delta_H$ 4.79 to $\delta_C$ 158.2) were confirmed. The relative configuration of the gluaiacylglyceryl moiety was deter-
mined as erythro by the Δδ of C-8'' and C-7'' (14.2, see Table 1) [7]. The absolute configurations for glucopyranosyl and gluacylglyceryl moiety were separately
determined as β-D and 7''R, 8''S according to Klyne rules [8] based on the following
data: [M]D = -248.9 for compound 9,
[M]D = -96.3 for tricin 4'-O-((7''R,8''S)-

- Recorded in DMSO-d6 at 600 and 150 MHz, respectively.

Table 1. ¹H and ¹³C NMR spectral data of compound 9 (δ in ppm, J in Hz).a.

| No. | δ_H | δ_C | No. | δ_H | δ_C |
|-----|-----|-----|-----|-----|-----|
| 2   | 160.7 | 4''  | 20 | 125.2 | 3'' |
| 3   | 6.97 (s) | 107.7 | 5'' | 6.70 (d, 8.0) | 114.7 |
| 4   | 177.1 | 6''  | 6.75 (br d, 8.2) | 119.4 |
| 5   | 158.2 | 7''  | 4.80 (overlap) | 72.2 |
| 6   | 6.91 (d, 2.2) | 103.4 | 8'' | 4.35 (dd, 8.9, 5.0) | 86.4 |
| 7   | 163.7 | 9''  | 3.74 (overlap, H_a), 3.50 (overlap, H_b) | 60.2 |
| 8   | 7.14 (d, 2.2) | 96.7 | 9   | 158.6 | 1'' |
| 10  | 109.3 | 2''  | 3.35 (overlap) | 73.6 |
| 1'  | 125.2 | 3''  | 3.35 (overlap) | 75.8 |
| 2'  | 7.32 (s) | 104.1 | 4'' | 3.17 (m) | 70.0 |
| 3'  | 153.0 | 5''  | 3.29 (overlap) | 77.6 |
| 4'  | 139.3 | 6''  | 3.50 (overlap, H_a), 3.72 (overlap, H_b) | 61.0 |
| 5'  | 153.0 | 7.32 (s) | 104.1 | 7'-OMe | 3.92 (s) | 56.2 |
| 6'  | 4.93 (br. s) | 111.0 | 3''-OMe | 3.74 (s) | 55.6 |
| 7'  | 147.0 | ΔδC8''-C7'' | 14.2 | 133.2 | 3''-OMe | 3.88 (s) | 56.5 |

Figure 2. Key ¹H–¹H COSY (——) and HMBC (—) correlations of compounds 9 and 10.
8''-guaiacylglycerol) ether [7], and 
[M]D = −66.3 for methyl β-D-glucopyranoside [9]. On biogenetic basis, it is reasonable for compound 9 to possess such absolute configurations by comparing with 1, and aglycone derivatives isolated from the same species [7]. Thus, the structure of compound 9 was determined as 4'-O-((7''R,8''R)-8''-guaiacylglycerol)-pleio- 
side B.

Compound 10 was isolated as yellowish needles with a molecular formula of C27H30O14 provided by HR-ESI-MS (m/z 577.1562 [M+2H]2). Its UV spectrum showed the characteristic absorption at 256 and 329 nm for the presence of flavonoid skeleton. The IR spectrum displayed the vibration bands at 3424 (OH), 1650 (conjugated CO), 1609, 1484, and 1457 (aromatic C=C) cm−1. The 1H NMR data (Table 2) indicated the presence of 5-OH [δ 13.47 (1H, s)], a 1,4-disubstituted phenyl group [δ 7.95 (2H, d, J = 8.8 Hz, H-2'/6')], 6.97 (2H, d, J = 8.8 Hz, H-3'/5')], two olefinic protons [δ 6.97 (1H, s, H-3), 6.88 (1H, s, H-8)], and two sugar moieties. In the 13C NMR spectrum (Table 2), 12 signals due to one hexopyranosyl and one 6-deoxy-hexopyr-

| No. | δH (ppm) | δC (ppm) |
|-----|----------|----------|
| 2   | 6.97 (s) | 164.3    |
| 3   | 6.97 (s) | 103.2    |
| 4   | 7.95 (d, 8.8) | 121.0 |
| 5   | 6.97 (d, 8.8) | 128.7 |
| 6   | 6.97 (d, 8.8) | 116.1 |
| 7   | 6.88 (s) | 94.7     |
| 8   | 6.97 (d, 8.8) | 116.1 |
| 9   | 6.97 (d, 8.8) | 128.7 |
| 10  | 6.97 (d, 8.8) | 116.1 |
| 1'  | 7.95 (d, 8.8) | 128.7 |
| 2'  | 6.97 (d, 8.8) | 116.1 |
| 3'  | 6.97 (d, 8.8) | 128.7 |
| 4'  | 6.97 (d, 8.8) | 116.1 |
| 5'  | 6.97 (d, 8.8) | 128.7 |
| 6'  | 6.97 (d, 8.8) | 116.1 |

*Recorded in DMSO-d6 at 600 and 150 MHz, respectively.*

Table 2. 1H and 13C NMR spectral data of compound 10 (δ in ppm, J in Hz).
159.4 (C-5), 111.3 (C-6), and 163.1 (C-7). According to Klyne rules [8], the structure of compound 10 was elucidated as apigenin 6-C-β-d-fucopyranosyl-7-O-β-d-glucopyranoside based on the following data: \([M]_D = -231.2\) for compound 10, \([M]_b = -66.3\) for methyl β-d-glucopyranoside [9], and \([M]_D = +172\) for apigenin 6-C-β-l-fucopyranoside [11].

Compounds 1–8 were identified as pleioside B (1) [12], pleioside A (2) [12], tricin 7-O-β-d-glucopyranoside (3) [13], farobin A (4) [14,15], liriodendrin (5) [16], tricin-7-O-neohesperidoside (6) [17], iso-orientin (7) [18], and (threo)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(E)-propenyl)-2,6-dimethoxyphenoxy]-propyl-β-d-glucopyranoside (8) [19,20] by comparison of their data with those reported.

The antioxidant activities of active fraction CE2B and the purified 10 compounds were investigated by assessing their roles in scavenging DPPH, ABTS, superoxide anion, and NO radicals (Table 3). Compounds 4 and 7 displayed significant antioxidant capacity, which led to a conclusion that they were the main antioxidant principles of N. affinis leaves.

### Table 3. Scavenging effects of fraction CE2B and compounds 1–10 on DPPH, ABTS, superoxide anion, and nitric oxide.

| Sample   | DPPH  | ABTS  | Superoxide anion | Nitric oxide |
|----------|-------|-------|------------------|--------------|
| CE2B     | 0.57a | 0.57a | 3.65a            | 0.91a        |
| 1        | 19.6%b| 51.7%b| 52.4%b           | 55.5%b       |
| 2        | Nullf | 13.5%b| Nullf            | Nullf        |
| 3        | Nullf | 41.7%b| 39.5%b           | 72.1%b       |
| 4        | 0.42a | 0.57a | 68.3%b           | 73.2%b       |
| 5        | Nullf | Nullf | Nullf            | 13.0%b       |
| 6        | Nullf | 49.5%b| 46.3%b           | 59.2%b       |
| 7        | 0.21a | 0.46a | 78.1%b           | 81.3%b       |
| 8        | 37.2%b| 65.9%b| 30.2%b           | 24.1%b       |
| 9        | 19.5%b| 1.10a | 46.2%b           | 55.6%b       |
| 10       | Nullf | Nullf | 50.0%b           | 40.8%b       |
| Vitamin C| 0.096a| 0.088a| _d               | _d           |
| Luteolin | _d   | _d   | 0.64a            | 0.34a        |

*a IC50 values (mg/ml).  
b Scavenging percentage at 2 mg/ml, except for compound 3 at 1 mg/ml.  
c No scavenging capacity observed.  
d No test.
plates precoated with 10–40 μm of silica gel GF254 from QHCC. An Agilent C18 column (4.6 mm × 250 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA) or a Welch C18 column (10 mm × 250 mm, 5 μm; Welch Materials Inc., Shanghai, China) was used on HPLC equipped with a Perkin-Elmer Series UV/VIS detector. All the other solvents were commercially purchased and distilled under normal atmospheric pressure prior to use.

3.2 Plant materials

The fresh leaves of *N. affinis* were collected in September 2011 from Pengzhou County, Chengdu City, Sichuan Province, China. The air-dried leaves were crushed and preserved at room temperature prior to use. A voucher specimen (2011–09) was identified by Prof. Fa-Ding Fu at Chengdu Institute of Biology, Chinese Academy of Sciences (CIBCAS), and was deposited in the Herbarium of CIBCAS.

3.3 Extraction and isolation

The treated *N. affinis* leaves (8 kg) were extracted with 60% ethanol at 70°C (3 × 801, 1.5 h each time). After removing the solvent under reduced pressure, the crude extract (CE, 960 g) was suspended in water (21), and extracted successively with EtOAc (3 × 21) and n-BuOH (3 × 21) to give EtOAc (CE1, 85 g), n-BuOH (CE2, 257 g), and water (CE3, 617 g) fractions. The isolation was performed guided by the antioxidant activity. The fraction CE2 with significant radical-scavenging capacity (IC50: 1.78 mg/ml on DPPH and 0.72 mg/ml on ABTS) was subjected to D101 macroporous resin column eluted with EtOH–H2O (0:100 to 100:0) to give four fractions (CE2A to CE2D). The more active fraction CE2B (IC50: 0.57 mg/ml on DPPH and 0.57 mg/ml on ABTS; 45 g) was subjected to CC (silica gel, 200–300 mesh) with gradient elution of CHCl3–MeOH (12:1 to 0:1, v/v) to give compound 2 (4 mg) and nine subfractions (CE2B1–CE2B9) based on TLC analyses. Fraction CE2B3 (4.35 g) was further separated over self-packed ODS column eluted with CH3OH–H2O (40:60, v/v) to give compounds 1 (8 mg) and 3 (2.5 mg), and six subfractions (CE2B3A–CE2B3F). Sub-fraction CE2B3C was separated by HPLC (Welch C18 column; 208 nm) with CH3CN–H2O (20:80, v/v; 4 ml/min) as solvents to get a fraction with retention time of 16.0 min, which was further separated by HPLC (Agilent C18 column; 254 nm) eluted with CH3CN–H2O (16:84, v/v; 1.5 ml/min) to afford compound 8 (tR 23.7 min; 7 mg). Subfraction CE2B3E was separated by HPLC (Welch C18 column; 208 nm) eluted with CH3CN–H2O (16:84, v/v; 1 ml/min) to a fraction with retention time of 16.0 min, which was further separated by HPLC (Agilent C18 column; 208 nm) taking CH3CN–H2O (16:84, v/v; 1 ml/min) as solvents to yield compound 9 (tR 62.0 min; 1.1 mg). The separation of fraction CE2B5 (1.8 g) over Sephadex LH-20 column eluted with MeOH gave compound 5 (5 mg). Fraction CE2B7 (5.23 g) was divided into nine subfractions (CE2B7A–CE2B7I) over Sephadex LH-20 column eluted with MeOH. Compound 7 (5 mg) was precipitated from subfraction CE2B7H. Subfraction CE2B7F was divided into CE2B7F1 (tR 21.0 min) and CE2B7F4 (tR 64.3 min) by HPLC (Welch C18 column; 208 nm) with CH3CN–H2O (24:76, v/v; 3 ml/min) as solvents. Fraction CE2B7F1 was then separated by HPLC (Agilent C18 column; 208 nm) with solvents CH3CN–H2O (18:82, v/v; 1 ml/min) to a fraction with retention time of 14.0 min, from which compound 10 (2.3 mg) was obtained by recrystallization (CH3OH–H2O, 1:1, v/v). The purification of fraction CE2B7F4 by HPLC (Agilent C18 column; 208 nm) with CH3OH–H2O (40:60, v/v; 1.5 ml/min) as solvents gave compound 6 (tR 71.0 min; 6 mg). Fraction CE2B8 (2.37 g) was separated over self-packed ODS
column eluted with CH$_3$OH–H$_2$O (30:70, v/v) to give subfraction CE2B8B, which was further separated over Sephadex LH-20 column eluted with MeOH to afford compound 4 (4 mg).

3.3.1 4’-O-((7R, 8S)-8’-guaiacylglyceryl)-pleioside B (9)

Yellow powder. $[\alpha]_D^{20}$ $-35.45$ (c 0.11, MeOH). UV (MeOH): $\lambda_{\text{max}}$ (log $\varepsilon$): 249 (3.9), 329 (4.1) nm. IR (KBr): $\nu_{\text{max}}$ 3436, 2925, 1634, 1600, 1496, 1456, 1423, 1352, 1244, 1180, 1125, 1071, 840, 811, 776, 735 cm$^{-1}$. For $^1$H and $^{13}$C NMR spectral data, see Table 1. HR-ESI-MS: $m/z$ 703.2244 [M$+\mathrm{H}^+$] (calcd for C$_{34}$H$_{39}$O$_{16}$, 703.2233); 725.2074 [M$+\mathrm{Na}^+$] (calcd for C$_{34}$H$_{38}$NaO$_{16}$, 725.2052).

3.3.2 Apigenin 6-C-$\beta$-D-fucopyranosyl-7-O-$\beta$-D-glucopyranoside (10)

Yellowish needles (MeOH–H$_2$O, 1:1, v/v). M.p. 220–223°C. $[\alpha]_D^{20}$ $-40$ (c 0.12, MeOH). UV (MeOH): $\lambda_{\text{max}}$ (log $\varepsilon$): 256 (4.5), 329 (4.5) nm. IR (KBr): $\nu_{\text{max}}$ 3424, 1650, 1609, 1484, 1457, 1349, 1246, 1183, 1076, 837, 776, 743 cm$^{-1}$. For $^1$H and $^{13}$C NMR spectral data, see Table 2. HR-ESI-MS: $m/z$ 577.1562 [M$-\mathrm{H}^-$] (calcd for C$_{27}$H$_{29}$O$_{14}$, 577.1563).  

3.4 Antioxidant assays

The DPPH scavenging assay was determined using a method reported previously [21], and the ABTS scavenging assay according to the method described by González et al. [22]. Vitamin C was used as a positive control. Superoxide anion-scavenging assay was performed according to the NADH–PMS–NBT (nicotinamide adenine dinucleotide (reduced form)-phenazine methosulfate–nitroblue tetrazolium chloride) method with luteolin as the positive control [23]. The detailed procedures of these experiments were described in our article reported before [24].

3.4.1 Nitric oxide-scavenging assay

In this study, nitric oxide radical-scavenging capacity was measured according to a previously reported method with minor modification [25]. Briefly, to the well of a 96-well plate containing 10 µl of samples or corresponding solvent (DMSO or me thanol, negative control) was added 100 µl of sodium nitroprusside solution (6 mM) in phosphate-buffered saline (0.2 M; pH 7.4). The mixture was then incubated under light (25 W tungsten lamp) for 180 min at room temperature. Griess reagent (100 µl), prepared freshly by mixing stock solution A [1% sulfanilamide (Tianjin Bodi Chem. Inc., Ltd, Tianjin, China) in 5% phosphoric acid] and stock solution B [0.1% naphthylethylenediamine dihydrochloride (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China)] with an equal volume, was added to each well, followed by incubation for 15 min at room temperature. Absorbance was then measured at 546 nm using a Varioskan Flash Reader. The nitric oxide radical-scavenging capacity (%) was calculated as

$$\text{Scavenging ratio} = \frac{1 - (A_e - A_s)}{A_c} \times 100\%,$$

where $A_e$ is the $A_{546}$ in the presence of sample, $A_s$ is the $A_{546}$ of sample, and $A_c$ is the $A_{546}$ of negative control solution. Luteolin was used as a positive control. Moreover, all analyses were done in triplicate.

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