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# Isolation and Identification of Compounds Responsible for Antioxidant Capacity of *Euryale ferox* Seeds

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Supporting Information

**ABSTRACT:** *Euryale ferox* seed is consumed medicinally or for food in China. The present study revealed it to contain significant antioxidant activity, which may be associated with its medical applications as a proteinuria inhibitor of diabetic nephropathy. This study resulted in the identification of 3 new sesquineolignans, named euryalins A−C (**1**−**3**), and 16 known compounds, which were all first isolated from this plant apart from 5,7,4-trihydroxy-flavanone. The antioxidant potential of the partial isolates was evaluated using the DPPH radical scavenging assay and mesangial cellular assay. Compounds **2**, *rel*-(2α,3β)-7-*O*-methylcedrusin (**4**), syringylglycerol-8-*O*-4-(sinapyl alcohol) ether (**5**), and (+)-syringaresinol (**7**) were found to be most active on DPPH assay, whereas compounds **2**, **4**, **7**, (1*R*,2*R*,5*R*,6*S*)-2-(3,4-dimethoxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane, and buddlenol E could significantly inhibit high glucose-stimulated reactive oxygen species production in mesangial cells. The results suggested that *E. ferox* seed could be considered as an excellent source of natural antioxidants and is useful in the prevention of diabetic nephropathy.

**KEYWORDS:** *Euryale ferox*, Nymphaeaceae, lignans, antioxidant activity

## INTRODUCTION

Diabetes mellitus is taking on epidemic proportions around the world because of the rapid change in lifestyle including changed diets and more sedentary work. It is predicted that the patients of diabetes mellitus will double in the period between 2000 and 2030.[1,2] Diabetes mellitus is an independent risk factor of many diseases such as cardiovascular diseases, stroke, and kidney diseases.[3,4] Accumulating evidence has demonstrated that diabetes mellitus is the leading cause of diabetic nephropathy, which accounts for nearly half of all new diabetes mellitus patients.[2] Diabetic nephropathy is a dangerous disease that can finally cause end-stage renal failure. Prevention of the development of diabetic nephropathy has received great attention in recent years. Accumulating research suggests that advanced glycation end product (AGE) formation and AGE-caused oxidative stress play critical roles in the pathogenesis of diabetic nephropathy.[5−7] Therefore, strategies designed to target oxidative stress may exert therapeutic effects on the progression of diabetic nephropathy.[7,8]

Due to the capability of antioxidants to attenuate oxidative stress in cells and aid in the prevention and treatment of many human diseases, the search for antioxidants has attracted much attention in the past decades, and many medicinal plants are considered to have great antioxidant potential.[9] *Euryale ferox* Salisb. (Nymphaeaceae), an aquatic plant, is the only species of the genus *Euryale* native to eastern Asia and has been found from India to Korea and Japan. In China, *E. ferox* is mainly produced in Guangxi, Shandong, Jiangsu, Hunan, and Anhui provinces.[10] The young stalks and rhizomes of this plant are edible, the seeds, locally known as Qianshi in Chinese as well as cock's head, are consumed medicinally or as food.[11] Qianshi is thought to be associated with the channels of the spleen and kidneys. Its main function is to strengthen the spleen and stop chronic diarrhea, to nourish the kidneys, and to stop excessive leucorrhea and prevent premature ejaculation.[11] In southern China, the seeds are served as food by adding them to soup or porridge. Notably, recent clinical studies showed Qianshi or preparations containing Qianshi, taking a formula consisting of Qianshi and the fruits of *Rosa laevigata* as an example, are able to reduce proteinuria, a typical syndrome of diabetic nephropathy.[12−15] However, to date, little is known about the mechanism of the therapeutic effect. Previous papers revealed that the seeds contain tocopherol trimers,[16] cyclic dipeptides,[17] glucosylsterols and cerebrosides.[16,18] Biological

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studies indicated that the seed extract showed significant antioxidant activity and cardioprotective property by inducing TRP32 and Trx-1 protein expression and scavenging reactive oxygen species.[19] Most recently, cerebrosides from the seeds were shown to be cytotoxic in the brine shrimp lethality bioassay.[16] Despite studies on the seeds of the plant, there is no study showing a rational correlation between the representative compounds and the therapeutic effects of the seeds for reducing proteinuria. As a follow-up to our group's interest in the intervention of diabetic nephropathy by traditional Chinese medicine,[20] in the present study, we hypothesized that the function of the seeds for the treatment of proteinuria of diabetic nephropathy may be mediated by the compounds in the plant with antioxidant activity, because oxidative stress is recognized as a major cause of diabetic nephropathy. A chemical investigation was therefore undertaken, and antioxidant activities of the selected compounds were evaluated by DPPH assay and cellular assay against high glucose-stimulated reactive oxygen species production in mesangial cells.

## MATERIALS AND METHODS

**General Experimental Procedures.** Optical rotations were recorded on a Horiba SEPA-300 polarimeter. UV spectra were determined on a Shimadzu double-beam 210A spectrometer. IR spectra were measured on a Tensor 27 spectrometer with KBr pellets. NMR spectra were collected on a Bruker AV-400 or a DRX-500 spectrometer. EIMS were determined on a Finnigan-4510 spectrometer. ESIMS and HRESIMS were recorded with an API QSTAR Pulsar 1 spectrometer. Column chromatography (CC) was carried out on silica gel (200−300 mesh, Qingdao Marine Chemical Inc., People's Republic of China), RP-18 (40−60 $\mu$m, Daiso Co., Japan), MCI gel CHP 20P (75−150 $\mu$m, Tokyo, Japan), and Sephadex LH-20 (Amersham Biosciences, Sweden). Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph; the column used was a 250 mm × 4.6 mm i.d., 5 $\mu$m, Zorbax SB-$C_{18}$.

**Plant Material.** The seeds of *E. ferox* produced in Guangxi province were obtained from Yunnan Corp. of Materia Medica (YCMM), Kunming, Yunnan Province, People's Republic of China, in May 2009, and were authenticated by Hong-Yan Sun at YCMM. A voucher specimen (CHYX0317) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China of Kunming Institute of Botany.

**Extraction and Isolation.** The sun-dried and ground seed powders of *E. ferox* (50 kg) were extracted three times with 85% EtOH (90 L × 3) under reflux. After concentration, the extract (1.2 kg) was suspended in water, which was followed by successive partition with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc-soluble extract (55 g) was applied to a silica gel column chromatography (CC) and eluted with increasing amounts (2%) of MeOH in $CHCl_3$ and finally MeOH to furnish four fractions (A−D). Fraction C (9.1 g) was divided into three portions (C1−C3) by MCI gel CHP 20P eluted with gradient aqueous MeOH (10%−80%). Fraction C1 (300 mg) was submitted to a Sephadex LH-20 column ($CHCl_3$/MeOH, 6:4) to yield compounds **11** (3 mg) and **17** (28 mg). Fraction C2 (600 mg) was applied to preparative TLC ($CHCl_3$/$^i$PrOH, 30:1) followed by Sephadex LH-20 CC ($CHCl_3$/MeOH, 6:4) to produce compounds **10** (18 mg), **12** (10 mg), and a mixture containing compounds **8** and **14**. This mixture was further purified by semipreparative HPLC (MeOH/$H_2O$, 60%) to give pure **8** (30 mg), **14** (4 mg). Fraction C3 (115 mg) was fractionated by Sephadex LH-20 CC (MeOH) and purified by preparative TLC ($CHCl_3$/$Me_2CO$, 20:1) to give compounds **6** (12 mg) and **7** (6 mg). Fraction D (9.5 g) was submitted to MCI gel CHP 20P column and washed with gradient aqueous MeOH (15−80%) to provide five portions (D1−D5). Fraction D1 (306 mg) was separated by Sephadex LH-20 (MeOH) followed by preparative TLC ($CHCl_3$/$^i$PrOH, 25:1) to yield compounds **1** (20 mg), **5** (13 mg), and **16** (4 mg). Fraction D2 (142 mg) was purified by semipreparative HPLC (MeOH/$H_2O$, 55−70%) to yield compounds **9** (5 mg), **13** (16 mg), and **15** (6 mg). Silica gel CC of fraction D3 (35 mg) eluted with $CHCl_3$/$Me_2CO$ (25:1) to give **2** (5 mg). Fraction D4 (107 mg) was separated by Sephadex LH-20 CC (MeOH) and successive semipreparative HPLC (MeOH/$H_2O$, 75%) to yield compounds **3** (2.7 mg), **18** (14 mg), and **19** (5.4 mg). Fraction D5 (75 mg) was fractionated by RP-18 column (MeOH/$H_2O$, 25−50%) to afford two portions (D5a and D5b). Of these, fraction D5b was purified by silica gel CC ($CHCl_3$/MeOH, 25:1) to yield compound **4** (8 mg).

**Table 1. NMR Data for Compounds 1 and 2 (500 MHz for $^1$H and 100 MHz for $^{13}$C, in $CD_3OD$)**

| | 1 | | 2 | |
|---|---|---|---|---|
| position | $^{13}$C | $^1$H | $^{13}$C | $^1$H |
| 2 | 88.6 | 5.53 d (6.0) | 88.6 | 5.50 d (6.1) |
| 3 | 55.7 | 3.47 (m) | 55.6 | 3.42 m |
| 3a | 65.0 | 3.85 overlap, 3.75 overlap | 65.0 | 3.80 overlap, 3.72 overlap |
| 4 | 117.9 | 6.70 overlap | 117.9 | 6.69 s |
| 4a | 129.5 | | 129.6 | |
| 5 | 137.1 | | 137.0 | |
| 5a | 32.9 | 2.60 t (7.5) | 32.9 | 2.60 t (7.4) |
| 5b | 35.8 | 1.80 m | 35.8 | 1.79 m |
| 5c | 62.2 | 3.55 t (6.6) | 62.2 | 3.54 t (6.5) |
| 6 | 114.1 | 6.79 br s | 114.0 | 6.79 s |
| 7 | 145.2 | | 145.2 | |
| 7a | 147.4 | | 147.5 | |
| 1′ | 139.5 | | 137.2 | |
| 2′ | 103.8 | 6.70 overlap | 111.4 | 6.96 br s |
| 3′ | 154.8 | | 151.8 | |
| 4′ | 136.4 | | 148.6 | |
| 5′ | 154.8 | | 116.1 | 6.65 d (7.6) |
| 6′ | 103.8 | 6.70 overlap | 119.2 | 6.85 d (7.6) |
| 1″ | 131.1 | | 130.7 | |
| 2″ | 114.1 | 6.72 br s | 114.2 | 6.70 br s |
| 3″ | 148.7 | | 148.7 | |
| 4″ | 145.8 | | 145.9 | |
| 5″ | 115.9 | 6.66 d (7.8) | 123.0 | 6.64 d (7.8) |
| 6″ | 123.1 | 6.62 d (7.8) | 117.4 | 6.88 d (7.8) |
| 7″ | 38.1 | 2.94 dd (15.6, 6.4), 2.89 dd (15.6, 6.4) | 37.6 | 2.90 dd (15.4, 6.2), 2.84 dd (15.4, 6.2) |
| 8″ | 85.5 | 4.22 m | 83.1 | 4.39 m |
| 9″ | 63.5 | 3.55 overlap, 3.49 overlap | 63.8 | 3.67 dd (15.0, 5.5), 3.62 dd (15.0, 6.5) |
| 7-OMe | 56.8 | 3.86 s | 56.8 | 3.85 s |
| 3′-OMe | 56.6 | 3.75 s | 56.6 | 3.76 s |
| 5′-OMe | 56.6 | 3.75 s | | |
| 3″-OMe | 56.3 | 3.77 s | 56.6 | 3.74 s |

**Euryalin A (1).** **1** was obtained as w white solid: $[\alpha]_D^{23}$ −3.1 (*c* 0.27, MeOH); IR (KBr) $\nu_{max}$ 3427, 2932, 2856, 1723, 1595, 1516, 1502, 1463, 1423, 1327, 1273, 1235, 1214, 1141, 1124,1034 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 281 (3.82), 235 (sh), 205 (4.95); $^1$H and $^{13}$C NMR spectra, see Table 1; electron impact mass spectrometry

Table 2. NMR Data for Compound 3 (500 MHz for $^1$H and 100 MHz for $^{13}$C, in $CD_3OD$)

| position | $^{13}$C | $^1$H | position | $^{13}$C | $^1$H |
|---|---|---|---|---|---|
| 1 | 136.4 | | 8′ | 136.4 | 4.27 m |
| 2 | 154.5 | | 9′ | 154.5 | 3.91 dd (12.0, 5.2), 3.60 overlap |
| 3 | 104.8 | 6.71 overlap | 1″ | 104.8 | |
| 4 | 134.7 | | 2″ | 134.7 | 6.81 s |
| 5 | 104.8 | 6.71 overlap | 3″ | 104.8 | |
| 6 | 154.5 | | 4″ | 154.5 | |
| 7 | 131.3 | 6.51 d (15.7) | 5″ | 131.3 | 6.69 d (8.0) |
| 8 | 129.9 | 6.29 dt (15.7, 5.5) | 6″ | 129.9 | 6.65 d (8.0) |
| 9 | 63.6 | 4.18 overlap | 7″ | 63.6 | 2.96 dd (15.4, 5.6), 2.89 dd (15.4, 5.6) |
| 1′ | 138.7 | | 8″ | 138.7 | 4.18 overlap |
| 2′ | 105.3 | 6.71 overlap | 9″ | 105.3 | 3.54 overlap, 3.48 dd (14.2, 4.2) |
| 3′ | 154.3 | | 2-OMe | 56.5 | 3.81 s |
| 4′ | 136.1 | | 6-OMe | 56.5 | 3.81 s |
| 5′ | 154.3 | | 3′-OMe | 56.6 | 3.79 s |
| 6′ | 105.3 | 6.71 overlap | 5′-OMe | 56.6 | 3.79 s |
| 7′ | 74.1 | 4.92 d (5.4) | 3″-OMe | 56.3 | 3.80 s |

(EIMS) $m/z$ 570 $[M]^+$; HR-EIMS $m/z$ 570.2468 $[M]^+$ (calcd for $C_{31}H_{38}O_{10}$, 570.2465).

**Euryalin B (2).** **2** was obtained as a white solid: $[\alpha]_D^{23}$ −2.4 ($c$ 0.22, MeOH); IR (KBr) $\nu_{max}$ 3431, 2925, 2854, 1620, 1513, 1462, 1426, 1271, 1139, 1032 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 282 (3.90), 228 (sh, 4.31), 205 (4.89); $^1$H and $^{13}$C NMR spectra, see Table 1; electron impact mass spectrometry (EIMS) $m/z$ 540 $[M]^+$; HR-EIMS $m/z$ 540.2353 $[M]^+$ (calcd for $C_{30}H_{36}O_9$, 540.2359).

**Euryalin C (3).** **3** was obtained as a white gum: $[\alpha]_D^{25}$ −9.3 ($c$ 0.22, MeOH); IR (KBr) $\nu_{max}$ 3442, 2935, 2848, 1590, 1515, 1462, 1421, 1330, 1227, 1125, 1020 cm$^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 271 (4.20), 228 (sh), 206 (4.81); $^1$H and $^{13}$C NMR spectra, see Table 2; electron spray ionization mass spectrometry (ESIMS) (positive-ion mode) $m/z$ 639 $[M + Na]^+$; HR-EIMS $m/z$ 616.2504 $[M]^+$ (calcd for $C_{32}H_{40}O_{12}$, 616.2520).

**Biological Activity.** *DPPH Radical Scavenging Assay.* The DPPH assay was carried out as previously described.[21] L-Ascorbic acid and gallic acid were used as positive controls, and reaction mixtures containing an EtOH solution of 200 $\mu$M DPPH (100 $\mu$L) and 2-fold serial dilutions of the sample (dissolved in 100 $\mu$L of EtOH, with sample concentrations in the range of 2−1000 $\mu$g/mL) were placed in a 96-well microplate and incubated at 37 °C for 30 min. After incubation, the absorbance was read at 517 nm by an Emax precision microplate reader, and the mean of three readings was obtained. Scavenging activity was calculated by the following equation:

$$\%\ \text{scavenging activity} = 100 \times \frac{\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}}$$

The $SC_{50}$ value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals.

*Analysis of Intracellular Reactive Oxygen Species Production.* The measurement of intracellular reactive oxygen species production was carried out in rat glomerular mesangial cells (HBZY-1, Life-Science Academy of Wuhan University, Wuhan, China) as previously described.[20] To examine the effect of the compounds, mesangial cells were pretreated with the indicated concentration (1 or 10 $\mu$M) of the compounds at 37 °C for 1 h and then exposed to either 5.6 mM (normal glucose, NG) or 25 mM (high glucose, HG) D-glucose [22] for 3 h using a Neubauer chamber. Fluorescence intensity was determined immediately by a flow cytometer (excitation $\lambda$ = 488 nm, emission $\lambda$ = 515 nm, BD FACSCalibur system, Franklin Lakes, NJ). The reactive oxygen species production was expressed as fluorescence intensity relative to control cells incubated in normal glucose and normalized by total cell protein contents for each experimental group of cells.[23]

*Statistical Analysis.* All of the experiments were performed in three replications. Continuous variables, expressed as mean ± SD, were compared using one-way ANOVA. Pairwise comparisons were evaluated by the Student−Newman−Keuls procedure or Dunnett's T3 procedure when the assumption of equal variances did not hold. The Dunnett procedure was used for comparisons between the reference group and other groups. A two-tailed $p$ value of <0.05 was considered to be statistically significant. Statistical analyses were conducted with SPSS 13.0.

## RESULTS AND DISCUSSION

**Structure Elucidation of Compounds.** Known compounds were identified as *rel*-(2α,3β)-7-*O*-methylcedrusin (**4**),[24] syringylglycerol-8-*O*-4-(sinapyl alcohol) ether (**5**),[25] (1*R*,2*R*,5*R*,6*S*)-2-(3,4-dimethoxyphenyl)-6-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**6**),[26] (+)-syringaresinol (**7**),[27] coniferyl aldehyde (**8**),[28] ω-hydroxypropioguaiacone (**9**),[29] *trans*-*p*-hydroxycinnamaldehyde (**10**),[30] *p*-hydroxybenzaldehyde (**12**),[31] *p*-hydroxybenzyl alcohol (**13**),[32] 4-hydroxybenzylethyl ether (**14**),[33] *p*-hydroxyphenethyl alcohol (**15**),[34] pyrogallic acid (**16**),[35] 5,7,4-trihydroxyflavanone (**18**),[17] and buddlenol E (**19**)[36,37] (Figure 1) by comparison of their spectroscopic data to previously reported values. Simple compounds **11** and **17** were each directly identified as 4-ethoxyphenol and 4-ethoxyphenol 2-methoxybenzene-1,3-diol by NMR and MS experiments. To our knowledge, except for 5,7,4-trihydroxyflavanone, all of the other known compounds were isolated from the seeds for the first time.

Compound **1** had the molecular formula $C_{31}H_{38}O_{10}$ deduced from the HR-EIMS. The IR spectrum indicated the presence of hydroxy (3427 cm$^{-1}$) and aromatic (1595, 1516, 1502, 1463, 1423 cm$^{-1}$) groups. The UV spectrum revealed absorptions at 235 and 281 nm. The $^1$H NMR spectrum (Table 1) of **1** showed four methoxy groups and two sets of aromatic protons at $\delta$ 6.70 (1H, overlap, H-4) and 6.79 (1H, brs, H-6) and at $\delta$ 6.72 (1H, brs, H-2″), 6.66 (1H, d, $J$ = 7.8, H-5″), and 6.62 (1H, d, $J$ = 7.8 Hz, H-6″). Additionally, a signal at $\delta$ 6.70 (2H, s, overlap, H-2′

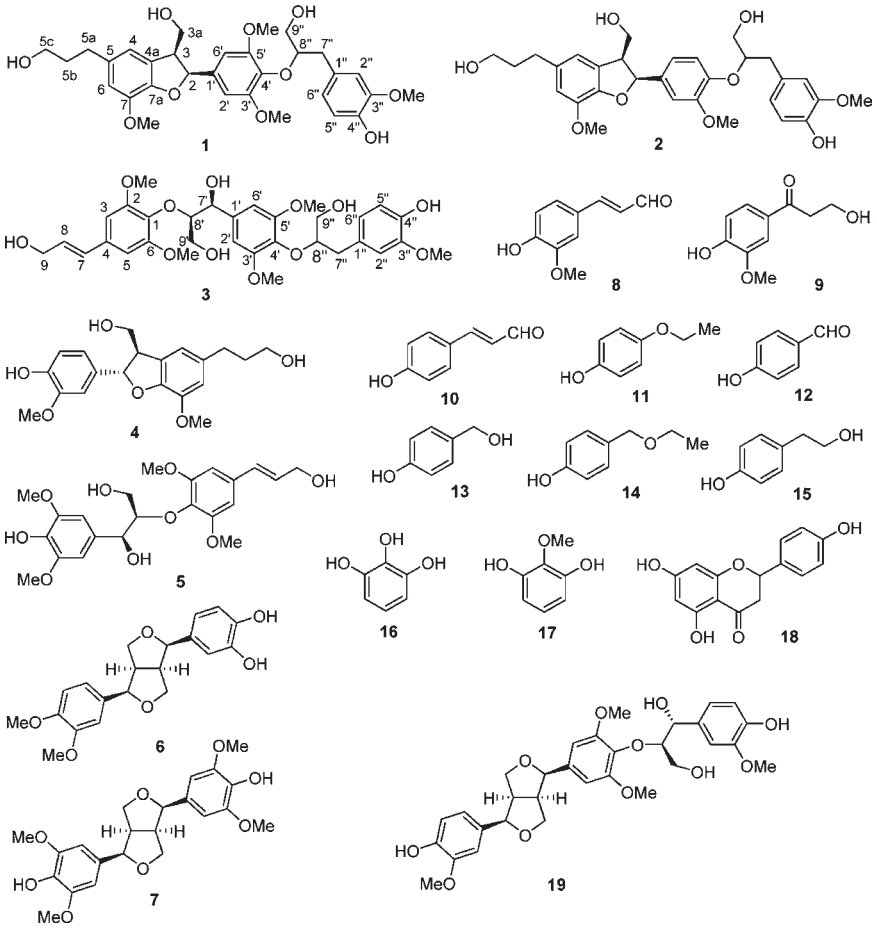

**Figure 1.** Structures of compounds **1**−**19**.

and H-6′) was observed in the $^{1}$H NMR spectrum. The $^{13}$C NMR spectrum displayed 18 signals (two overlapped ones), indicative of three aromatic rings in the molecule. The $^{1}$H and $^{13}$C NMR (Table 1) signals at $\delta_H$ 2.94 (1H, dd, $J$ = 15.6, 6.4 Hz, Ha-7″), 2.89 (1H, dd, $J$ = 15.6, 6.4 Hz, Hb-7″), 4.22 (1H, m, H-8″), and 3.55 (1H, overlap, Ha-9″), 3.49 (1H, overlap, Hb-9″) and $\delta_C$ 38.1 (C-7″), 85.5 (C-8″), and 63.5 (C-9″) indicated the presence of a 7″-dehydroxyglyceryl residue. The spectroscopic data of **1** were similar to those of acernikol,[38] the difference being that one hydroxyl attached at C-7″ of acernikol was missing in **1**. This conclusion was confirmed by $^{1}$H−$^{1}$H COSY cross peaks of H-7″/H-8″/H-9″ and HMBC correlations of H-7″ with C-1″, C-2″, and C-6″ (Figure 2). ROESY correlation between OMe-3″ and H-2″ further confirmed the substituted pattern of the benzene ring. The relative configuration of the dihydrofuran ring in **1** was clarified by ROESY experiments (Figure 2), which showed a correlation between H-2 and H-3, revealing a *cis*-relationship of these two protons. The absolute configuration at C-8″ remained undetermined. Therefore, compound **1** was defined as 7″-dehydroxyacernikol and named euryalin A.

Compound **2** was assigned the molecular formula $C_{30}H_{36}O_9$ from its HR-EIMS. The IR spectrum showed absorption bands at 3431, 1662, and 1426 cm$^{-1}$, indicative of hydroxy and aromatic moieties. The UV spectrum indicated absorptions at 228 (sh) and 282 nm. The IR, UV, and $^{1}$H and $^{13}$C NMR spectroscopic data of **2** resembled with those of **1**. The only difference was that OMe-5′ in **1** was absent in **2**, which resulted in an ABX coupling pattern in **2** [$\delta_H$ 6.96 (1H, br s, H-2′), $\delta_H$ 6.65 (1H, d, $J$ = 7.6 Hz, H-5′), and $\delta_H$ 6.85 (1H, brd, $J$ = 7.6 Hz, H-6′)]. Analysis of $^{1}$H−$^{1}$H COSY, HMQC, HMBC, and ROESY experiments confirmed the planar structure and allowed unambiguous assignments of NMR data for compound **2**. The relative configuration at the dihydrofuran ring was determined by the ROESY experiments. The ROESY correlation between H-2 and H-3 revealed a *cis*-type isomer. The absolute configuration at C-8″ remained unknown. The above evidence allowed compound **2** to be determined as 7″-dehydroxy-5′-demethoxyacernikol and named euryalin B.

Compound **3** exhibited a [M]$^{+}$ ion peak at $m/z$ 616.2504 in the HR-EIMS, in accordance with the molecular formula $C_{32}H_{40}O_{12}$. The IR absorptions at 3442, 1590, 1515, 1462, and 1421 cm$^{-1}$ indicated the presence of hydroxy and aromatic moieties. The $^{13}$C NMR spectrum showed 18 aromatic carbons (4 overlapped ones) corresponding to three benzene rings. The similarity of the NMR data of **3** to those of **5** indicated that they are analogues, except for the addition of a 7-dehydroxyguaiacylglyceryl moiety in the molecule of **3** [$\delta_H$ 2.96 (1H, dd, $J$ = 15.4, 5.6 Hz, Ha-7″), 2.89 (1H, dd, $J$ = 15.4, 5.6 Hz, Hb-7″), 4.18 (1H, overlap, H-8″), 3.54 (1H, overlap, Ha-9″, 3.48 (1H, dd, $J$=14.2, 4.2 Hz, Hb-9″), $\delta_C$ 38.0 (C-7″), 85.6 (C-8″), 63.4 (C-9″)]. The HMBC correlation between H-8″ and C-4′ and consideration of their chemical shifts suggested the connection of C-4′ and C-8″ via an ether linkage. The coupling constant between H-7′ and H-8′ is 5.4 Hz, indicative of an *erythro* isomer.[26,39] So far, the


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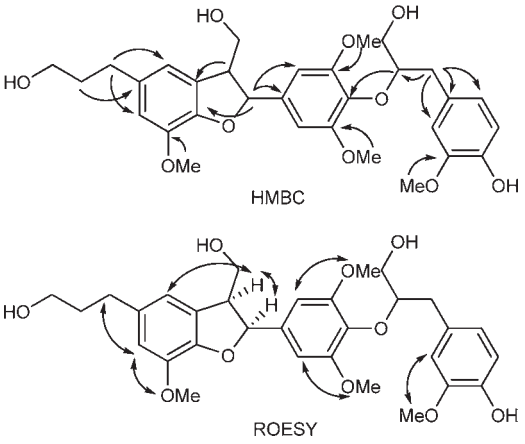



**Figure 2.** Key HMBC and ROESY correlations for compound **1**.

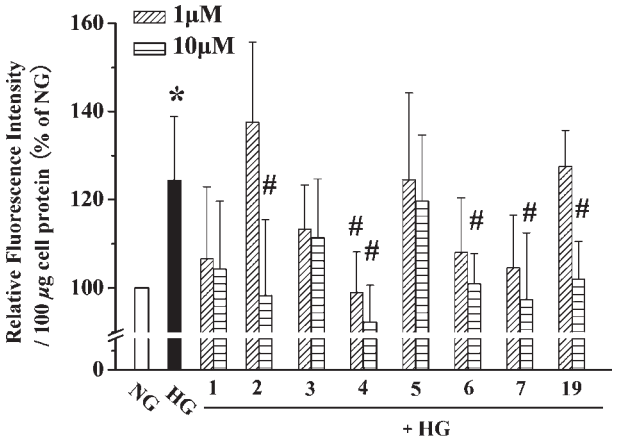
**Table 3. DPPH Radical Scavenging Activity[a] of the Extracts, Fractions,[b] and Isolates**

| sample | DPPH ($SC_{50}$) | sample | DPPH ($SC_{50}$) |
|---|---|---|---|
| EtOH extracts | 68.7 | **9** | 103.1 |
| petroleum ether fraction | 29.0 | **10** | 2085.3 |
| EtOAc fraction | 20.4 | **11** | 768.7 |
| *n*-BuOH fraction | 78.8 | **12** | 495.2 |
| **1** | 237.2 | **13** | 1317.7 |
| **2** | 6.8 | **14** | 699.1 |
| **3** | 162.3 | **15** | 830.4 |
| **4** | 10.4 | **16** | 26.2 |
| **5** | 10.2 | **17** | 41.1 |
| **6** | 27.5 | **18** | 209.7 |
| **7** | 12.9 | ascorbic acid | 20.8 |
| **8** | 477.5 | gallic acid | 10.7 |

[a] $SC_{50}$ values of the extracts and fractions, and the isolates are represented as $\mu$g/mL and $\mu$M, respectively. [b] 5 g of powdered *E. ferox* seeds was exhaustively extracted by 95% EtOH (10 mL × 3) under reflux followed by evaporation to yield EtOH extracts; partition of EtOH extracts suspension in water by petroleum ether, EtOAc, and *n*-BuOH afforded respective fraction for DPPH assay.

absolute configuration at C-8″ has not been determined. Consequently, compound **3** was identified as 4′-*O*-(2-guaiacyl-2,3-propanediol)syringylglycerol-8-*O*-4-(sinapyl alcohol) ether and given the name euryalin C.

**Antioxidant Activity Evaluation of Compounds.** Because *E. ferox* seeds are always used to treat kidney diseases including diabetic nephropathy and oxidative stress has been considered to be one cause of diabetic nephropathy, we evaluated the antioxidant property of the isolated compounds of *E. ferox* seeds. Although many methods have been developed to measure the antioxidant activity, the DPPH radical scavenging assay has been considered to be the most reliable one and is used widely.[9] Therefore, the antioxidant capability of the extracts, fractions, and isolated compounds of the *E. ferox* seeds were first examined by DPPH assay using ascorbic acid and gallic acid as positive controls. As shown in Table 3, the EtOAc fraction displayed the most potent activity ($SC_{50}$ = 20.4 $\mu$M). Compounds **2**, **4**, **5**, and **7** exhibited strong effects against DPPH assay with $SC_{50}$ values of 6.8, 10.4, 10.2, and 12.9 $\mu$M, respectively, comparable to that of the positive control (gallic acid or ascorbic acid). Interestingly,




ANOVA, $p < 0.01$; *$p < 0.05$ vs NG, #$p < 0.05$ vs HG

**Figure 3.** Lignan compounds inhibited high glucose-induced ROS production in mesangial cells. Data are expressed as mean ± SD of three independent experiments. ANOVA, $p < 0.001$; ∗, $p < 0.05$ versus NG; #, $p < 0.05$ versus HG.

although the structures of compounds **1** and **2** are similar, except that compound **1** contains an additional methoxy group, the antioxidant activities of compounds **1** and **2** are very different. These data suggested that the presence of OMe-5′ might decrease the inhibitory activity. To further evaluate the antioxidant capacity of lignans (i.e., compounds **1**−**6** and **19**), mesangial cells were treated with lignans before stimulation by high glucose. As shown in Figure 3, high glucose-stimulated reactive oxygen species production was significantly inhibited by compounds **2**, **4**, **6**, **7**, and **19** at the concentrations of 10 $\mu$M. The above results indicated that the diverse components in the seeds with antioxidants activity and their synergistic antioxidant effects may be important reasons for the therapeutic effect of the seeds to reduce proteinuria, and *E. ferox* seeds would be good candidates in the search for natural antioxidants useful for the prevention of diabetic nephropathy.

## ■ ASSOCIATED CONTENT

**Supporting Information.** $^{1}$H and $^{13}$C NMR spectra of compounds **1**−**3**, HMBC and ROESY correlations of compounds **1** and **2**, references to known compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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