Phytochemistry Letters 16 (2016) 1–7

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

New lignans with neuroprotective activity from *Adelostemma gracillimum*

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form 4 February 2016
Accepted 12 February 2016
Available online 26 February 2016

Keywords:
Adelostemma gracillimum
Cortical neurons
NMDA excitotoxicity
Lignans
Neolignans
Acetophenones

ABSTRACT

Adelostemma gracillimum is an herb used as nourishing and roborant drugs and in the treatment of convulsions in children. To date, a few molecular constituents have been isolated from the root of this herb and chemically characterized, but their biological activities have never been reported. Here, we demonstrate that the crude extract of *A. gracillimum* (AGE) can protect primary cortical neurons against *N*-methyl-D-aspartate (NMDA)-induced cytotoxicity. Further fractionations of AGE led to the isolation of four novel lignans (**1–4**), two known lignans (**5**,**11**), and five known acetophenones (**6–10**); their structures were elucidated by comparison with related literature, extensive analyses of NMR spectroscopy and high-resolution mass spectrometry. Of the eleven isolates, lignans **2**, **3** and **5** exhibit significant neuroprotection against NMDA-induced cell death. This is the first report of isolating lignans with neuroprotective activity from *A. gracillimum*.

1. Introduction

Adelostemma gracillimum (Wall. ex Wight) Hook. f. & Tsiang is a liana plant widely distributed in southwest China and Myanmar. Its root is used as a nourishing roborant and in the treatment of convulsions in children (Mu et al., 1992). The crude glycoside-containing extract of *A. gracillimum* (AGE) has been demonstrated to afford protection against seizures in rat models (Mu et al., 1992; Raza and Iqbal Choudhary, 2000). To date, only 4 aglycones from the hydrolyzed crude glycosides (Mu et al., 1992) and 6 glycosides (Gao et al., 2009) isolated from the root of this herb have been chemically characterized, but the biological activities of these chemical constituents have not been investigated. To identify new natural agents for treating neurological diseases that are characterized by neuronal cell death, we have conducted a phytochemical study of *A. gracillimum*. Herein, we report the unidentified neuroprotective property of AGE against *N*-methyl-D-aspartate (NMDA)-induced cytotoxicity and the isolation of four novel (**1–4**) and two known (**5**, **11**) lignans, and five known acetophenones (**6–10**) from AGE. The structural elucidation of the four novel lignans is described. Lignans **2, 3** and **5** offer significant neuroprotection against NMDA-induced cell death. This is the first report of isolating lignans from *A. gracillimum* which have neuroprotective property.

2. Results and discussion

2.1. Neuroprotective effect of A. gracillimum extract

AGE from *A. gracillimum* was prepared as described previously with some modifications (Mu et al., 1992). The consistency of the preparations was assured by their HPLC fingerprints (Fig. S1 and Table S1 of Supplementary information). The neuroprotective property of AGE against NMDA-induced cytotoxicity was investigated in cultured neurons. Quantitative analysis showed that AGE 10–50 μg/mL reduced cell death by ~60–80%, indicating AGE can significantly protect neurons from NMDA excitotoxicity (Fig. 1a). Memantine, which also exhibits neuroprotective effect (Lipton, 2006) was used as a positive control in this assay. In addition, AGE

Abbreviation: AGE, crude extract from *Adelostemma gracillimum*.

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http://dx.doi.org/10.1016/j.phytol.2016.02.011
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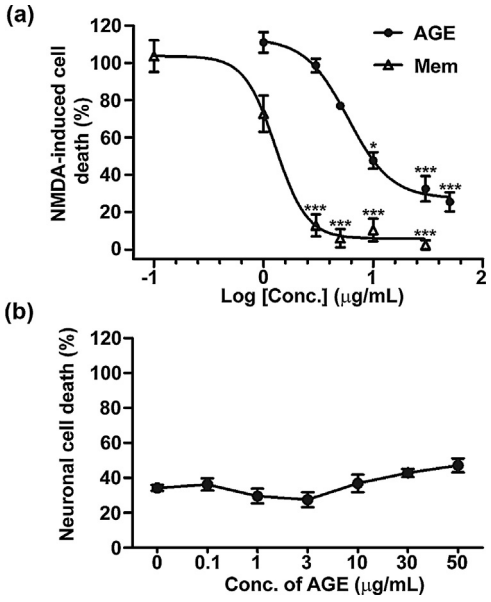

Fig. 1. Dose-dependent neuroprotective effect of extract from *Adelostemma gracillimum*. (a) Cell death in cultured rat cortical neurons was induced by 20 μM NMDA. Normalized cell death in the presence of various concentrations of AGE and memantine (control) are shown. Statistical analysis was performed by two-way ANOVA followed by Bonferroni post hoc tests, $^{*}p < 0.05$, $^{***}p < 0.005$. (b) AGE 0.1–50 μg/mL was not toxic to cortical neurons upon 24 h treatment as revealed by the relatively unchanged normalized cell death despite increasing concentration.

0.1–50 μg/mL did not induce any cytotoxic effect on cortical neurons compared to that of the vehicle control (Fig. 1b).

2.2. Structural elucidation

Four novel (**1–4**) and two known (**5**, **11**) lignans, and five known acetophenones (**6–10**) were isolated from AGE. The structural elucidations of the isolated compounds are described as follows.

Compound **1** was isolated as a light yellow oil, with a molecular formula of $C_{19}H_{22}O_5$ determined by the high-resolution mass spectrometry (HRMS) at *m/z* 353.1380 ($[M + Na]^+$) and *m/z* 313.1465 ($[M + H–H_2O]^+$). The ^1H and ^{13}C NMR spectra of **1** imply the presence of six aromatic protons belonging to two independent benzene rings (rings A, B). Careful analyses of 2D NMR spectra involving ^1H–^1H COSY, HSQC and HMBC (Fig. 3) successfully assign the six aromatic protons with signals at δ_H 7.05 (1H, s, H-2), 6.78 (1H, d, J = 8.0 Hz, H-5), and 6.84 (1H, d, J = 8.0 Hz, H-6) to ring A, and signals at δ_H 6.85 (1H, d, J = 1.5 Hz, H-2′), 6.83 (1H, d, J = 8.0 Hz, H-5′), and 6.72 (1H, dd, J = 8.0, 1.5 Hz, H-6′) to ring B, indicating the presence of two 1,3,4-trisubstituted aromatic rings in **1**. A methoxyl group at δ_H 3.85 (3H, s)/δ_C 56.4, attached to the C-3 position of ring A, is evidenced by the HMBC correlation between δ 3.85 and 148.8 (C-3), together with the NOESY spectrum showing correlation between the methoxyl group and H-2 (Fig. 4). The presence of a propylene glycol unit was deduced from the ^1H–^1H COSY spectrum displaying sequential cross peaks from H-7 (δ_H 4.88) to H-9 (δ_H 1.15) via H-8 (δ_H 4.43), and the linkage of propylene glycol unit at C-1 of ring A was indicated by the correlations of H-7 to both C-2 (δ_C 111.3)/C-6 (δ_C 120.4) in HMBC. Meanwhile, the linkage of a propenyl moiety at C-1′ of ring B is established by the HMBC correlations between H-7′ (δ_H 6.26) and C-6′ (δ_C 118.8)/C-2′ (δ_C 113.8), which is supported as well by the NOE correlations between H-7′ and H-2′ in the NOESY spectrum of **1**. The arrangement of an OH group at C-3′, suggested by the 1,3,4-trisubstituted pattern of aromatic ring B and the molecular formula of **1** being $C_{19}H_{22}O_5$, is clearly supported by the correlations of from H-2′ to C-3′ (δ_C 149.0)/C-6′/C-7′ (δ_C 131.9), along with H-5′ to C-3′ in HMBC. The NMR spectroscopic data of **1** resembles that of known lignan machilin C (Hada et al., 1988; Shimomura et al., 1987; Sung et al., 2001), with the major difference being that **1** has an OH group at C-3′, while a —OCH_3 at C-3′ in machilin C (Table 1). The *erythro*-configuration of protons H-7 and H-8 is suggested by both the small coupling constant of $J_{7,8}$ = 3.5 Hz (~3.0/8.0 for *erythro*/*threo*, respectively) and the carbon chemical shift of C-9 at δ_C 13.6 (~13.0/17.0 for *erythro*/*threo*, respectively) (Braga et al., 1984; Hada et al., 1988; Shimomura et al., 1987; Sung et al., 2001), being consistent with that of machilin C (Hada et al., 1988; Shimomura et al., 1987; Sung et al., 2001). Furthermore, the coupling constant between H-7′ and H-8′ (15.5 Hz) indicates the *trans* configuration of the olefinic double bond in the 1′-propenyl moiety. Therefore, the structure of **1** is elucidated to be *erythro*-3-methoxyl-3′,4,7-trihydroxyl-(7′*E*)-(8-*O*-4′)-neolignan-7′-en, as shown in Fig. 2.

Table 1
^1H (500 MHz) and ^{13}C NMR (75 MHz) data of compounds **1–4** in CD_3OD.

No	1		2		3		4	
	d_H (mult., J in Hz)	d_C	d_H (mult., J in Hz)	d_C	d_H (mult., J in Hz)	d_C	d_H (mult., J in Hz)	d_C
1	–	133.6	–	133.2	–	134.5	–	133.9
2	7.05 (s)	111.3	6.67 (s)	105.4	6.88 (s)	110.1	6.60 (s)	103.8
3	–	148.8	–	149.2	–	148.8	–	149.3
4	–	146.8	–	135.9	–	147.3	–	136.3
5	6.78 (d, 8.0)	115.8	–	149.2	6.74 (d, 8.0)	116	–	149.3
6	6.84 (d, 8.0)	120.4	6.67 (s)	105.4	6.75 (d, 8.0)	119.3	6.60 (s)	103.8
7	4.88 (d, 3.5)	76.2	4.58 (d, 8.0)	79.8	5.14 (d, 4.5)	92.6	5.16 (d, 5.0)	92.8
8	4.43 (m)	81.1	3.98 (m)	86.7	3.69 (m)	47.3	3.71 (m)	47.4
9	1.15 (d, 6.0)	13.6	1.13 (d, 6.5)	17.9	1.30 (d, 7.0)	20.7	1.31 (d, 6.5)	20.7
1′	–	133.9	–	132.2	–	123.8	–	124.1
2′	6.85 (d, 1.5)	113.8	6.50 (s)	102.5	–	132.4	–	132.5
3′	–	149	–	154.7	–	153.5	–	153.6
4′	–	145.6	–	135.8	–	153.4	–	153.6
5′	6.83 (d, 8.0)	117.2	–	152	6.85 (d, 8.0)	115	6.87 (d, 8.5)	115.1
6′	6.72 (dd, 8.0, 1.5)	118.8	6.51 (s)	107.9	6.83 (d, 8.0)	117.4	6.74 (d, 8.5)	117.6
7′	6.26 (d, 15.5)	131.9	6.28 (d, 16.0)	132.2		204.8	–	205.3
8′	6.08 (m)	124.5	6.16 (m)	125.7	2.60 (s)	32.1	2.60 (s)	32.2
9′	1.82 (d, 6.5)	18.5	1.84 (d, 7.5)	18.5	–	–	–	–
3-OCH_3	3.85 (s)	56.4	3.84 (s)	56.8	3.79 (s)	56.3	3.79 (s)	56.7
5-OCH_3	–	–	3.84 (s)	56.8	–	–	3.79 (s)	56.7
3′-OCH_3	–	–	3.80 (s)	56.3	–	–	–	–

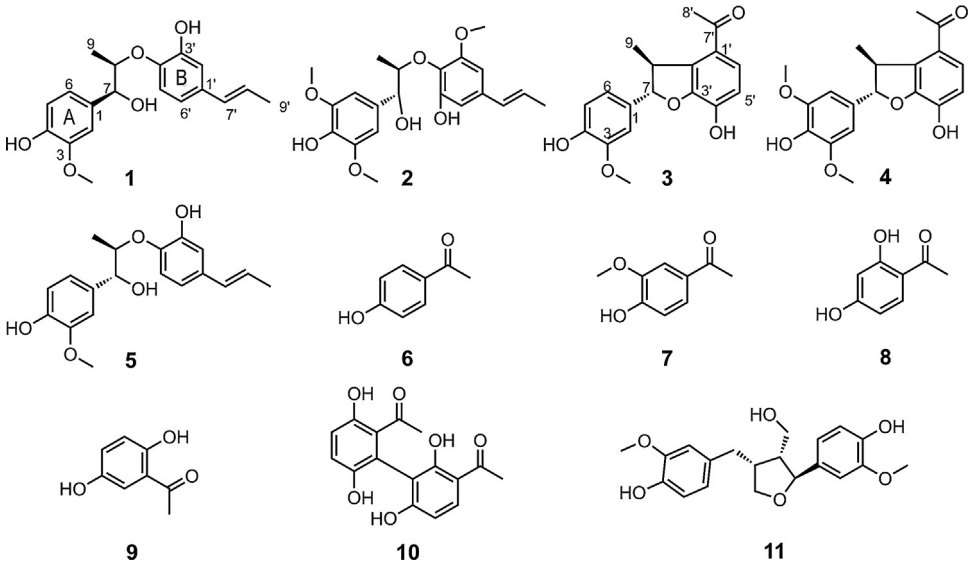

Fig. 2. Chemical structures of compounds **1–11**.

Compound **2** is isolated as an oil. Its molecular formula, $C_{21}H_{26}O_7$, is determined on the basis of the molecular ion peaks $[M+H]^+$ at *m/z* 391.1743, $[M+Na]^+$ at *m/z* 413.1576, and $[M+H-H_2O]^+$ at *m/z* 373.1660 in HRMS. Comparison of the ^1H and ^{13}C NMR spectroscopic data of **2** with those of **1** (Table 1) reveals their structural similarities, indicating **2** is a neolignan as well. The ^1H NMR spectrum exhibits resonances characteristic of four aromatic protons appearing as three singlets at δ_H 6.67 (2H, H-2/H-6), δ_H 6.50 (1H, H-2′), and 6.51 (1H, H-6′), thus demonstrating the presence of two 1,3,4,5-tetrasubstituted benzene rings in compound **2**. The same chemical shifts of H-2/H-6 in the ^1H NMR, together with the same chemical shifts of C-2/C-6 (δ_C 105.4), C-3/C-5 (δ_C 149.2) in the ^{13}C NMR, indicate that one tetrasubstituted benzene ring (ring A) is axially symmetric. The ^1H and ^{13}C NMR data show that **2** contains three methoxyl groups at δ_H 3.80 (3H, s)/δ_C 56.3 (3′-OCH_3) and δ_H 3.84 (6H, s)/δ_C 56.8 (3-OCH_3/5-OCH_3). HMBC correlation between 3′-OCH_3 and C-3′ (δ_C 154.7), in combination with the NOE correlation between 3′-OCH_3 and H-2′, reveal that the methoxyl group at δ_H 3.80 is arranged at C-3′, while the assignment of other two methoxyl groups at C-3/C-5, resulting in an axial symmetry in ring A, is determined by the correlations between 3-OCH_3 and C-3 in the HMBC, and between H-2 and 3-OCH_3 in the NOESY (Figs. 3 and 4). Furthermore, the ring B, with a propenyl group at C-1′, is deduced from the ^1H–^1H COSY data and the HMBC correlations from H-7′ (δ_H 6.28) to both C-2′ (δ_C 102.5) and C-6′ (δ_C 107.9). Finally, the connection of C-8 and C-4′ via an oxygen atom, is successfully established by the correlation between H-8 (δ_H 3.98) and C-4′ (δ_C 135.8) in the HMBC. The *trans* configuration is assigned to the olefinic double bond in ring B by the large coupling constant of $J_{7',8'}$ being 16.0 Hz. Interestingly, different to that of **1**, the relative configuration between H-7 and H-8 in **2** is assumed to be *threo* instead of *erythro* based on the coupling constant of $J_{7,8}$ = 8.0 Hz and the carbon chemical shift of C-9 at δ_C 17.9 (Braga et al., 1984; Hada et al., 1988; Shimomura et al., 1987; Sung et al., 2001). Therefore, the structure of **2** was determined to be *threo*-4,5′,7-trihydroxyl-3,3′,5-trimethyoxyl-(7′*E*)-(8-*O*-4′)-neolignan-7′-en (Fig. 2).

Compound **3** is obtained as a pale yellow gum. The molecular formula, $C_{18}H_{18}O_5$, is determined on the basis of the ion peak $[M+H]^+$ at *m/z* 315.1241 in HRMS. The ^1H and ^{13}C NMR

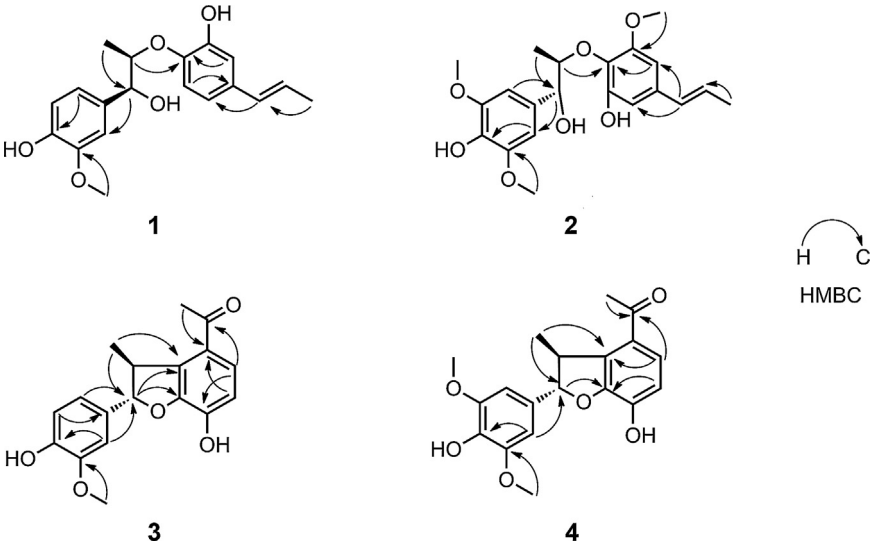

Fig. 3. Key HMBC correlations in compounds **1–4**.

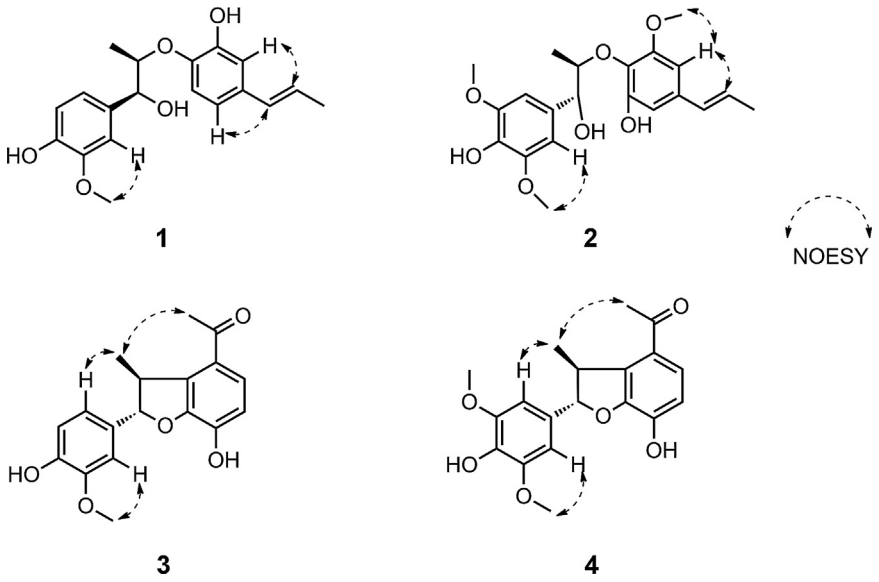

Fig. 4. Key NOESY correlations in compounds **1–4**.

spectroscopic data of **3** (Table 1) are similar to those of **1**–both having two independent benzene rings (A/B), one methoxy group, and one doublet methyl—with the major difference being that **3** has one carbon less than **1**, indicating that **3** is a norneolignan, like bombasin (Wu et al., 2008). The assignment of the methoxy group and hydroxyl group in ring A to C-3/C-4, respectively, is justified by the distinguished HMBC correlations of H-2 (δ_H 6.88) with C-7 (δ_C 92.6)/C-4 (δ_C 147.3), H-5 (δ_H 6.74) with C-1 (δ_C 134.5)/C-3 (δ_C 148.8), and H-6 (δ_H 6.75) with C-7, and is corroborated by the correlation of H-2 with 3-OCH_3 (δ_H 3.79) in the NOESY spectrum (Fig. 4). Whereas, for ring B, the diagnostic correlation of H-5′ (δ_H 6.85) with H-6′ (δ_H 6.83) in the ^{1}H-^{1}H COSY spectrum and the coupling constant ($J_{5',6'}$ = 8.0 Hz) between them, support that ring B is a 1,2,3,4- tetrasubstituted benzene derivative. Furthermore, the presence of one acetyl group and one hydroxyl group at C-1′/C-4′, respectively, is established by the correlations of H-8′ (δ_H 2.60) with C-1′ (δ_C 123.8), H-6′ with C-4′ (δ_C 153.4)/C-7′(δ_C 204.8), and H-5′ with C-1′/C-3′ (δ_C 153.5) in HMBC. Finally, the connection of these two rings via a dihydrofuran fragment to furnish the planar structure of **3**, is constructed by the diagnostic correlations of H-7 (δ_H 5.14) with C-9 (δ_C 20.7)/C-2′ (δ_C 132.4)/C-3′, H-9 (δ_H 1.30) with C-2′ in HMBC, in combination with the sequential correlations of from H-7 toward H-9 in the ^{1}H–^{1}H COSY spectrum. The relative configuration of the protons at C-7 and C-8 is deduced to be *trans* by the NOESY spectrum showing correlation of H-7 with CH_3-9. However, compared to the *trans* bombasin (Wu et al., 2008), **3** has a relatively small coupling constant between H-7 and H-8 (4.5 Hz); this could be due to the stereo-hindrance effect caused by the adjacent acetyl group at C′-1, which is supported by the correlations of CH_3-9 with both H-8′ and H-6 in NOESY. Thus, the structure of compound **3** was determined to be (7*S**,8*S**)-3-methoxyl-4,4′-dihydroxyl-1′-acetyl-3′,7-epoxy-2′,8-neolignan (Fig. 2), which is a relatively rare 9′-norneolignan.

Compound **4** is obtained as a pale yellow gum. The molecular formula of compound **4** is determined to be $C_{19}H_{20}O_6$ on the basis of the HRMS peaks $[M+H]^+$ at *m/z* 345.1340 and $[M+Na]^+$ at *m/z* 367.1162. The ^{1}H, ^{13}C NMR spectra of compound **4** are similar to those of **3** (Table 1), supporting that **4** is a norneolignan as well. The ring A of **4**, being the same as that of **2**, readily identified by the distinguished signals at δ_H 6.60 (s, 2H, H-2/H-6), δ_H 3.79 (s, 6H, 3-OCH_3/5-OCH_3) in the ^{1}H NMR spectrum, is clearly established by the HMBC showing correlations from H-2 to C-3 (δ_C 149.3)/C-4 (δ_C 136.3)/C-7 (δ_C 92.8), together with the correlation between the 3-OCH_3 and H-2 in the NOESY (Figs. 3 and 4). Careful examination of the 1D, 2D NMR data indicate that the ring B of **4** is the same as that of **3**, which is evidenced by the ^{1}H, ^{1}H COSY displaying correlation between H-5′ (δ_H 6.87) and H-6′ (δ_H 6.74), and the diagnostic correlations from H-8′ (δ_H 2.60) to C-1′ (δ_C 124.1), H-5′ to C-1′/C-3′ (δ_C 153.6), H-6′ to C-2′ (δ_C 132.5)/C-4′ (δ_C 153.6) in the HMBC. Similar to **3**, the connection of rings A and B via a dihydrofuran moiety, was constructed by the correlations of H-7 (δ_H 5.16) with C-2′/C-3′, H-9 (δ_H 1.31) with C-2′/C-7 (δ_C 92.8) in the HMBC. The NOESY spectrum of **4** displays an NOE interaction between H-7 and CH_3-9, supporting the assignment of the 7*S**,8*S** relative configuration, being the same as **3**. Thus, the structure of compound **4** is determined to be (7*S**,8*S**)-3,5-dimethoxyl-4,4′-dihydroxyl-1′-acetyl-3′,7-epoxy-2′,8-neolignan (Fig. 2).

The remaining compounds (**5–11**) were identified by comparing their spectroscopic data (MS, ^{1}H and ^{13}C NMR data) and the specific optical rotation data with those of related literature. They were identified as Callislignan B (**5**) (Rattanaburi et al., 2012), piceol (**6**) (Li et al., 2008; Sigstad et al., 1993; Yeo and Kim, 1997), acetovanillone (**7**) (Li et al., 2008; Xiao and Parkin, 2007; Yeo and Kim, 1997), resacetophenone (**8**) (Yeo and Kim, 1997), quinacetophenone (**9**) (Calanasan and MacLeod, 1998; Yeo and Kim, 1997), cynandione A (**10**) (Lin et al., 1997; Yeo and Kim, 1997) and (+)-laricresinol (**11**) (Lee et al., 2007; Okuyama et al., 1995).

2.3. Neuroprotective effects of isolated compounds

All eleven compounds isolated from AGE were subjected to neuroprotection assay using rat primary cortical neurons challenged with NMDA. Compounds, **2**, **3** and **5** showed significant neuroprotective effect (Table 2). Compound **2** at 30 μM reduced NMDA-induced neuronal death by ~35%. Compounds **3** and **5** are more potent and reduced neuronal death by ~30% at 10 μM and ~50% at 30 μM. Furthermore, **2**, **3** and **5** did not show cytotoxicity in primary cortical neurons (Table S2). Interestingly, all three neuroprotective compounds are lignans, and two have novel structures (**2** and **3**). Since the extent of neuroprotection AGE could provide is ~75% at 50 μg/mL (Fig. 1a), individual active compounds seem less potent (assuming abundance is <1%). It is possible that

Table 2
Neuroprotective properties of isolated compounds from AGE.

Compound	Neuronal cell death (%) at tested concentration		
	1 μM	10 μM	30 μM
1	95.5 ± 3.1	87.1 ± 0.3	71.9 ± 6.4
2	96.1 ± 3.5	72.2 ± 8.8	65.8 ± 2.9*
3	72.6 ± 4.3	71.1 ± 2.9*	50.5 ± 7.1**
4	91.8 ± 7.4	91.3 ± 6.2	80.7 ± 6.0
5	85.3 ± 4.7	65.4 ± 5.9*	54.6 ± 4.7**
6	≥100	98.3 ± 5.0	≥100
7	95.8 ± 2.2	99.1 ± 0.7	≥100
8	92.7 ± 0.6	≥100	≥100
9	≥100	≥100	≥100
10	87.7 ± 0.2	88.0 ± 0.1	93.8 ± 1.9
11	93.1 ± 0.6	86.3 ± 3.0	90.1 ± 1.8
Control	0.1 μM	1 μM	5 μM
Memantine	95.6 ± 1.9	76.6 ± 2.1*	12.5 ± 2.8**

* $p < 0.05$.
** $p < 0.01$.

more active compounds have yet to be identified, or/and compounds **2**, **3** and **5** can act synergistically. Nevertheless, AGE and its neuroprotective constituents should be further investigated for their mechanisms of action and potential to alleviate symptoms of diseases such as stroke, neurodegenerative disorders and epilepsy whose underlying causes involve NMDA receptor mediated neuronal death (Dong et al., 2009; Parsons and Raymond, 2014).

3. Experimental

3.1. General experimental procedures

Optical rotation $[\alpha]_D^{20}$ was measured by a PerkinElmer 241 polarimeter in MeOH at room temperature. NMR spectra including ^1H, ^{13}C, correlation spectroscopy (COSY), (Nuclear Overhauser effect spectroscopy) NOESY, (Heteronuclear single-quantum correlation spectroscopy) HSQC, and (Heteronuclear multiple-bond correlation spectroscopy) HMBC were recorded on a Varian Mercury 300 or 500 MHz NMR spectrometer. Compounds were dissolved in CD_3OD (3–10 mg/mL), and spectra were acquired at room temperature (~22 °C). Chemical shifts (δ) were reported in ppm using the solvent peak as reference (^1H—3.31 ppm; ^{13}C—49.05 ppm). High resolution electrospray ionization mass spectrometry (HRESIMS) and electrospray ionization mass spectrometry (ESIMS) spectra were measured by a Finnigan MAT LCQ ion trap mass spectrometer and an Applied Biosystems QSTAR XL™ equipped with turbo-ion spray, respectively. Column chromatography was performed using silica gel (40–63 μm, Merck, Darmstadt, Germany), macroporous adsorption resin (D101, Minsheng™, Tianjin, China), and LiChroprep RP-C18 gel (40–63 μm, Merck, Darmstadt, Germany). Preparative RP HPLC was carried out on a Waters 2545 Binary Gradient Module pump system equipped with 2996 Photodiode Array Detector using X-bridge C18 column (19 × 150 mm, 5 μm, Waters). Semi-prep HPLC was performed using the same Waters system with a Nova-Pak C-18 column (7.8 × 300 mm, 6 μm, Waters). Thin-layer chromatography was performed on Merck aluminum sheets coated with 0.25 mm silica gel 60 F_{254} and visualized under ultraviolet light or by spraying with 10% H_2SO_4 in ethanol followed by heating.

3.2. Plant materials

The roots of *A. gracillimum* were collected in Zhongdian of Yunnan province, People's Republic of China, in July of 1997, 2009, and 2010, and authenticated by Prof. Quanzhang Mu (Kunming Institute of Botany, Chinese Academy of Science). The herb samples were air-dried and then powdered. Voucher specimens (A2176) were deposited in the Molecular Neuroscience Center at The Hong Kong University of Science and Technology. Extracts from three batches of *A. gracillimum* were prepared and called AGE-1 to AGE-3, respectively.

3.3. Extraction, analysis and isolation of compounds

The dried roots of *A. gracillimum* (5.5 kg) were powdered and immersed in ethanol for 30 min at 1:5 (w/v). The extraction was carried out by refluxing 1.5 h and repeated twice. The extracts were combined and dried, and then suspended in water at w/v 1:5 and partitioned using chloroform at v/v 1:1 three times. The chloroform extract was then defatted with petroleum ether (60–80 °C) by reflux for 1 h at 1:10 (w/v), and the insoluble residues were collected, dried, and re-dissolved in 70% aqueous ethanol solution (1:10 w/v). The insoluble substance was removed by filtration. The solution was evaporated to dry, and the refined extract (230 g) was obtained as a yellow powder (AGE).

AGE was further fractionated by using macroporous absorption resin (D101) column chromatography and eluted consecutively with 30%, 60%, and 96% EtOH/H_2O to afford 3 fractions: I (7 g), II (35 g), and III (189 g). Only fractions I and III exhibited significant neuroprotective activity, and were subjected to silica gel column chromatography. 8 sub-fractions (A1-A8) were obtained from I and 22 sub-fractions (F1–F22) were obtained from III by gradient eluent starting with petroleum ether-EtOAc to EtOAc-$CHCl_3$ and finally to $CHCl_3$-MeOH. By RP-18 column chromatography eluted with a gradient solution of MeOH and H_2O, F6 was fractionated to give 10 sub-fractions. The sub-fractions were further separated by using RP-18 prep-HPLC with a gradient solution of CH_3CN/H_2O. F6-4 was first separated to 2 sub-fractions including F6-4-1, which was further purified by a second round of RP-18 prep-HPLC to afford compounds **1** (18.8 mg) and **5** (7.5 mg). Fractions F8 and F9 were fractionated by silica gel column chromatography eluted with a gradient solution of $CHCl_3$ and $(CH_3)_2CO$ to yield 9 and 8 fractions, respectively. By using RP-18 prep-HPLC, CH_3CN/H_2O gradient as an eluting system, F8-5 was further purified to afford compound **2** (4.8 mg); and F9-5 was fractioned by RP-18 prep-HPLC followed by RP-18 semi-prep HPLC purification to yield compounds **3** (7.7 mg) and **4** (3.5 mg). Fractions A3 and A4 were subjected to RP-18 prep-HPLC by using a gradient solution of CH_3CN-H_2O for further separation. Compounds **6** (55.6 mg), **7** (4.4 mg), and **8** (4.6 mg) were obtained from A4; **9** (10.8 mg), **10** (13.5 mg) and **11** (13.6 mg) were isolated from A3. The yield of compounds 1–11 are summarized in Table S3. All isolates were estimated to be over 90% pure based on their appearances in TLC and ^{13}C NMR spectra (see Supplementary information).

3.3.1. Compound ***1***

IUPAC name: 4-((1*S*,2*R*)-1-hydroxy-2-(2-hydroxy-4-((*E*)-prop-1-en-1-yl) phenoxy) propyl)-2-methoxyphenol; light yellow oil; $[\alpha]_D^{20}$ +16.6 (*c* 0.82, MeOH); UV (MeOH) λ_{max}: 259 nm; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS (positive-ion mode) at *m/z* 353.1380 $[M+Na]^+$ (calculated for $C_{19}H_{22}O_5Na$, 353.1365), *m/z* 683.2809 $[2M+Na]^+$(calculated for $C_{38}H_{44}O_{10}Na$, 683.2832), *m/z* 313.1465 $[M-H_2O+H]^+$ (calculated for $C_{19}H_{20}O_4H$, 313.1440).

3.3.2. Compound ***2***

IUPAC name: 2-(((1*R*,2*R*)-1-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl) propan-2-yl) oxy)-3-methoxy-5-((*E*)-prop-1-en-1-yl) phenol; viscous oil; $[\alpha]_D^{20}$ −5.3 (*c* 0.34, MeOH); UV λ_{max} (MeOH):

260 nm; ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS (positive-ion mode) at *m/z* 391.1743 $[M+H]^+$, 413.1576 $[M+Na]^+$ and *m/z* 373.1660 $[M+H-H_2O]^+$ (calculated for $C_{21}H_{26}O_7H$, 391.1757 and $C_{21}H_{26}O_7Na$, 413.1576).

3.3.3. Compound **3**

IUPAC name: 1-((2*S*,3*S*)-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-methyl-2,3-dihydrobenzofuran-4-yl) ethanone; pale yellow gum; $[\alpha]_D^{20}$ +2.3 (*c* 0.94, MeOH); UV λ_{max} (MeOH): 231 and 336 nm; ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS (positive-ion mode) at *m/z* $[M+H]^+$ 315.1241 (calculated for $C_{18}H_{18}O_5H$, 315.1232).

3.3.4. Compound **4**

IUPAC name: 1-((2*S*,3*S*)-7-hydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-3-methyl-2,3-dihydrobenzofuran-4-yl) ethanone; light yellow gum; $[\alpha]_D^{20}$ +8.7 (*c* 0.31, MeOH); UV λ_{max} (MeOH): 228 and 335 nm; ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS (positive-ion mode) at *m/z* $[M+H]^+$ 345.1340 and $[M+Na]^+$ 367.1162 (calculated for $C_{19}H_{20}O_6H$, 345.1338 and $C_{19}H_{20}O_6Na$, 367.1158).

3.3.5. Known compounds **5–11**

The IUPAC names of **5**–**11** are 4-((1*R*,2*R*)-1-hydroxy-2-(2-hydroxy-4-((*E*)-prop-1-en-1-yl) phenoxy) propyl)-2-methoxyphenol (**5**), 1-(4-hydroxyphenyl) ethanone (**6**), 1-(4-hydroxy-3-methoxyphenyl) ethanone (**7**), 1-(2,4-dihydroxyphenyl) ethanone (**8**), 1-(2,5-dihydroxyphenyl) ethanone (**9**), 1,1′-(2′,3,6,6′-tetrahydroxy-[1,1′-biphenyl]-2,3′-diyl) diethanone (**10**) and 4-((2*S*,3*R*,4*R*)-4-(4-hydroxy-3-methoxybenzyl)-3-(hydroxymethyl) tetrahydrofuran-2-yl)-2-methoxyphenol (**11**).

3.4. Cortical neuron culture and assays

Primary cortical neurons were prepared as described previously (Fu et al., 2007, 2010). Isolated cortical cells from E18 Sprague–Dawley rats were plated on a 48-well plate (Nunc) at 1.1×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27 (Life Technologies). AGE was dissolved at 10 mg/mL in 100% DMSO. To detect the possible cytotoxicity of AGE, cultured cortical neurons at 11 DIV were treated with various concentrations of AGE for 24 h and lactate dehydrogenase (LDH) release into the medium (Roche) was measured after 24 h incubation. Data are presented as average percentage ± SEM compared to 0.1% Triton X-100 as a control (set as 100% cytotoxicity).

Neuroprotection assay was performed as described previously with some modifications (Sun et al., 2003). Neurons at 11 DIV were treated with various concentrations of tested samples (e.g. AGE, isolated compounds) or 0.5% DMSO (vehicle) for 24 h, and the medium was changed to Locke's medium containing glycine for 15 min. Neurons were subsequently treated with 20 μM NMDA, to induce excitotoxicity and cell death, in the presence of tested samples (or vehicle) for 20 min. Some neurons that were treated with 0.5% DMSO were spared of NMDA treatment. Neurons were then grown in normal medium for 24 h and cell death was quantified by LDH release. Neuronal cell death (%) is calculated as: $100\%*(LDH_X-LDH_1)/(LDH_2-LDH_1)$, where LDH_X, LDH_1 and LDH_2 denote LDH release in the presence of extract/compound + NMDA, DMSO alone and DMSO + NMDA, respectively. Data are presented as average percentage ± S.E.M. and experiments were repeated at least 3 times with duplicate wells. Memantine (Sigma), a known neuroprotective compound (Lipton, 2006), was included for comparison.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

JZ and SG prepared the extract, isolated the compounds, elucidated the chemical structures and drafted the manuscript. EPST conducted neuroprotection assay. KWC coordinated experiments, assisted data analysis for neuroprotection assay and drafting the manuscript. GF conducted fingerprinting of extract and assisted in herb identification and structural elucidation. QM assisted in herb identification and extraction. FCFI and NYI planned and designed the experiments, as well as finalizing the manuscript.

Acknowledgements

We are grateful to Mr. Fangyuan Kang, Drs. Wei Zhang, Amy K. Fu and Yu Pong Ng for their critical reading of the manuscript. We would like to thank Ms. Venus WS Lee and Mr. Joe Yim for their excellent technical assistance. This study was supported in part by the Hong Kong Research Grants Council Theme-based Research Scheme (T13-607/12R), the National Key Basic Research Program of China (2013CB530900), the Innovation and Technology Fund for State Key Laboratory (ITCPT/17-9), the Shenzhen Peacock Plan, and the SH Ho Foundation. Last, we express our profound gratitude to the late Dr. Quanzhang Mu, who has offered us his generous guidance and whose pioneered work on the chemical and biological studies of *A. gracillimum* laid the foundation of this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.02.011.

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