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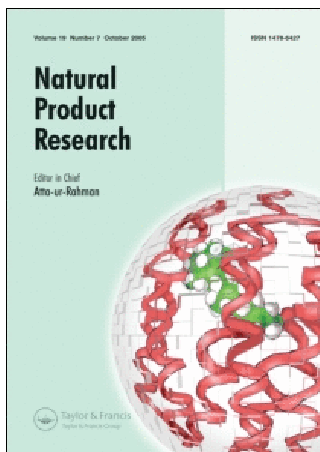

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Two ellagic acid glycosides from *Gleditsia sinensis* Lam. with antifungal activity on *Magnaporthe grisea*

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Two ellagic acid glycosides were isolated by bioassay-guided fractionation from the antimicrobial ethyl acetate fraction of the ethanol extract from *Gleditsia sinensis* spines, and identified as 3-*O*-methylellagic acid-4′-(5″-acetyl)-α-L-arabinofuranoside (**1**) and 3-*O*-methyl-ellagic acid-4′-*O*-α-L-rhamnopyranoside (**2**). Both compounds were isolated from this plant species for the first time, and **1** is a new compound. The two compounds showed significant antifungal activity against the spore germination of rice blast fungus *Magnaporthe grisea*, with an IC_{50} value of 13.56 $\mu g\,mL^{-1}$ for **1** and 16.14 $\mu g\,mL^{-1}$ for **2**.

Keywords: Leguminosae; *Gleditsia sinensis* Lam.; Spine; Ellagic acid glycoside; Antifungal activity

1. Introduction

Gleditsia sinensis Lam. (Leguminosae) is a medicinal plant widely distributed in China, and its spines have been used in traditional Chinese medicine for the treatment of various cancers, heart, vascular and infectious diseases [1–3]. Triterpenoids and monoterpenoids have been reported from this plant [4–7]. Our previous study showed that the ethanol extract of *G. sinensis* spines had antimicrobial activity [8]. The present study aimed to isolate and identify the antimicrobial compounds from *G. sinensis* spines based on bioassay-guided fractionation.

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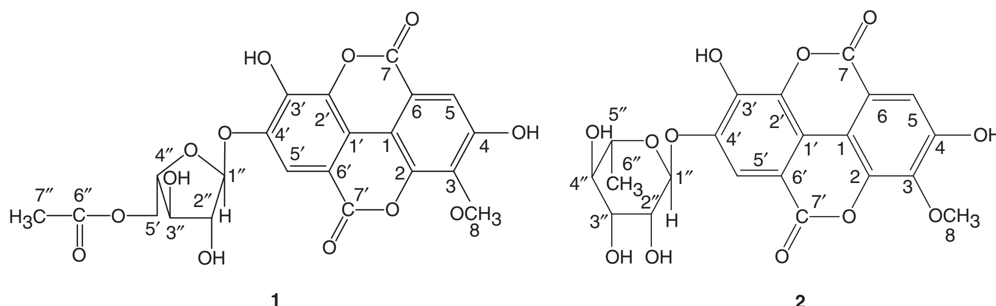
This report shows the isolation, structure elucidation and antifungal activity of 3-*O*-methylellagic acid-4′-(5″-acetyl)-α-L-arabinofuranoside (**1**) and 3-*O*-methylellagic acid-4′-*O*-α-L-rhamnopyranoside (**2**) from the ethyl acetate (EtOAC) fraction of the ethanol extract of *G. sinensis* spines. To our knowledge, the two compounds have not been isolated from *G. sinensis*, and **1** is a new ellagic acid glycoside.



2. Results and discussion

Column chromatography of the EtOAC fraction of the ethanol extract from the *G. sinensis* spines afforded compounds **1** and **2** which had the physicochemical properties shown in table 1. The molecular structures were elucidated based on their UV, IR, MS, and NMR spectral data.

Compound **1** was obtained as a white powder, with the characteristic UV spectrum of *O*-methylellagic acid [9]. Its IR spectrum exhibited peaks at 3367 cm^{-1} (–OH), 1751, 1701 cm^{-1} (lactone functions), and 1608 cm^{-1} (aromatic ring). Upon acid hydrolysis, each mole of **1** yielded one mole of arabinose. The ESI-MS of **1** showed an $[M-H]^-$ peak at $m/z = 489$, and an $[M+Na]^+$ peak at $m/z = 513$, and the HR-ESI-MS showed an $[M+Na]^+$ peak at $m/z = 513.0637$, which indicate the molecular formula $C_{22}H_{18}O_{13}$. The ^1H NMR spectrum of **1** showed two aromatic protons at δ 7.65 (1H, s) and δ 7.39 (1H, s), one methoxy group at δ 4.09 (3H, s), and one acetyl group at δ 1.98 (3H, s) (table 2).

The ^{13}C NMR spectrum of **1** exhibited 12 signals (table 2, C-1 to 6 and C-1′ to 6′) together with two carbonyl carbons at δ 160.5 and δ 160.6 due to α,β-unsaturated lactones, one methoxy carbon at δ 62.0, carbons of one acetyl group (δ 20.7 and δ 172.7), and five sugar carbons (table 2, C-1″ to 5″) whose chemical shifts matched those of arabinofuranose. The ^1H and ^{13}C NMR spectra suggested a mono-*O*-methylellagic acid structure of **1** by comparison of their chemical shifts with those of references [10,11].

Table 1. Physicochemical properties of compounds **1** and **2**.

Compound	Appearance	Melting point (°C)	IR(KBr)γ_{max} (cm^{-1})	UV (MeOH) λ_{max} (nm)
1	White powder	139–142	3367, 1751, 1701, 1608, 1577, 1496, 1107, 1076	250, 360
2	Yellow powder	300	3344, 1743, 1701, 1604, 1573, 1492	252, 364

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The HMQC spectrum of **1** showed correlation between H-5 proton signal (δ 7.39) and C-5 carbon signal (δ 112.9). Signal correlations were also observed between H-5′ (δ 7.65) and C-5′ (δ 113.0), H-8 (δ 4.09) and C-8 (δ 62.0), and H-7″ (δ 1.98) and C-7″ (δ 20.7). These proton-carbon signal correlations also suggest the existence of methoxy group, acetyl group and methylellagic acid moiety in the structure of **1**. The HMBC spectrum showed a long-range correlation between H-1″ signal (δ 5.64) and C-4′ signal (δ 147.7) (table 2), which indicates the position of the arabinose moiety at C-4′. The methoxy group was concluded to be at C-3 position based on another long-range correlation between H-8 (δ 4.09) and C-3 (δ 141.7). Other long-range correlations were observed between H-5 (δ 7.39) and C-3, C-4, C-6, and C-7, and between H-5′ (δ 7.65) and C-3′, C-4′, C-6′, and C-7′. The configuration of the anomeric carbon was concluded to be α from the *J*-value (1.5 Hz) in the ^{1}H NMR spectrum. Eventually, **1** was identified as 3-*O*-methylellagic acid 4′-(5″acetyl)-α-L-arabinofuranoside.

Compound **2** was obtained as a yellow powder, which had UV and IR spectra both similar to those of **1** (table 3). Its acid hydrolysis yielded one mole rhamnose per mole; its ESI-MS showed an $[M-H]^{-}$ peak at $m/z = 461$; its ^{1}H NMR spectrum showed two aromatic protons at δ 8.48 (1H, s) and δ 8.08 (1H, s), one methoxy group at δ 4.18 (3H, s). The ^{13}C NMR spectrum of **2** exhibited 12 signals (C-1 to 6 and C-1′ to 6′) together with two carbonyl carbons at δ 161.4 and δ 161.1 due to α,β-unsaturated lactones, one methoxy carbon at δ 62.9, and six sugar carbons (table 3, C-1″ to 6″) with chemical shifts matching those of rhamnopyranose. The ^{1}H and ^{13}C NMR spectra suggested a mono-*O*-methylellagic acid structure of **2** by comparison of their chemical

Table 2. The ^{1}H NMR and ^{13}C NMR data of compound **1** (in CD_3OD, δ values).

Position	δ_C	δ_H	HMBC correlations
1	112.9		
2	142.8		
3	141.7		
4	153.9		
5	112.9	7.39 (s)	114.4, 141.7, 153.9, 160.6
6	114.4		
7 (COO)	160.5		
8 (OCH_3)	62.0	4.09 (s)	141.7
1′	116.1		
2′	137.4		
3′	143.2		
4′	147.7		
5′	113.0	7.65 (s)	109.1, 116.1, 143.2, 147.7, 160.6
6′	109.1		
7′ (COO)	160.6		
Arabinose			
1″	108.7	5.64 (d, $J = 1.5$ Hz)	79.0, 82.8, 147.7
2″	82.8	4.35 (dd, $J = 1.5$, 4 Hz)	79.0, 85.3
3″	79.0	3.96 (dd, $J = 4$, 6 Hz)	65.2, 82.8
4″	85.3	4.23 (dt, $J = 3$, 6, 7 Hz)	65.2,
5″	65.2	4.16 (dd, $J = 7$, 11 Hz),	85.3, 172.7
		4.26 (dd, $J = 3$, 11 Hz)	85.3, 172.7
6″ (CO)	172.7		
7″ (CH_3)	20.7	1.98 (s)	172.7

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Table 3. The ^{1}H NMR and ^{13}C NMR data of compound **2** (in C_5D_5N, δ values).

Position	δ_C	δ_H	HMBC correlations
1	113.8		
2	144.4		
3	142.9		
4	155.9		
5	116.5	8.04 (s)	113.8, 115.9, 142.9, 155.9, 161.4
6	115.9		
7 (COO)	161.4		
8 (OCH_3)	62.9	4.18 (s)	142.9
1′	117.1		
2′	139.2		
3′	146.0		
4′	108.1		
5′	114.4	8.48 (s)	117.1, 146.0, 108.1, 109.3, 161.1
6′	109.3		
7′(COO)	161.1		
Rhamnose			
1″	103.7	6.43 (s)	73.5
2″	73.5	4.91 (br)	74.2
3″	74.2	4.77 (dd, $J = 9.6$, 3.2 Hz)	
4″	75.3	4.41 (t, $J = 9.6$ Hz)	20.0, 74.2
5″	73.1	4.61 (m)	
6″	20.0	1.66 (d, $J = 6.4$ Hz)	73.1, 75.3

Table 4. Inhibitory activity of the compounds **1** and **2** on *M. grisea* spore germination.

Sample	Inhibition regression equation ($Y = aX + b$)[a]	Correlation coefficient (R)	IC_{50} ($\mu g\, mL^{-1}$)
1	$Y = 8.111X - 4.183$	0.9963	13.56
2	$Y = 10.904X - 8.170$	0.9886	16.14
Amphotericin B	$Y = 10.467X - 3.803$	0.9994	6.94

[a] $Y = aX + b$, where Y is the inhibitory probit value and X is logarithmic concentration of the compound.

shifts with those in the references [12,13]. Based on the HMQC and HMBC spectral data, and comparison of their NMR spectral data with those in the literature [12,13], **2** was identified as 3-*O*-methylellagic acid 4′-*O*-α-L-rhamnopyranoside.

The antifungal tests showed that both compounds **1** and **2** had significant inhibitory activity against the spore germination of rice blast fungus, *Magnaporthe grisea* (table 4). The median effective inhibitory concentration (IC_{50}) values against the spore germination were 13.56 $\mu g\, mL^{-1}$ for **1** and 16.14 $\mu g\, mL^{-1}$ for **2**, respectively.

Ellagic compounds are a large group of polyphenols widely distributed in plants. There has been increasing interest in the wide range of biological and pharmacological activities of these phytochemicals including antioxidative, anticancer, and antimicrobial activities [14]. The results from the present study may provide the chemical basis for the resistance of *G. sisnensis* plant to microbes in the natural environment. Further studies are needed to understand the structure–activity relationship and antimicrobial mechanisms.

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3. Experimental

3.1. *General experimental procedures*

NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin, Billerica, MA, USA) at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The chemical shifts were expressed in ppm as δ values relative to tetramethylsilane (TMS) as an internal standard. ESI-MS were performed on a Thermo Finnigan LCQ mass spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA), and HR-ESI-MS were performed on a VG Auto Spec-3000 mass spectrophotometer (Micromass, Manchester, UK). Melting points were measured with a YRT-3 micro-melting point device (uncorrected, Tianjin, China). IR spectra were recorded on a Bio-Rad FTS-65A FTIR spectrometer (Bio-Rad, USA) with KBr pellets. Column chromatography was performed with silica gel 60 (70–230 mesh) (Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Biosciences, Inc.). Thin layer chromatography (TLC) was performed on pre-coated silica gel F_{254} plates (Qingdao Marine Chemical Inc.), and compound spots were visualized by spraying vanillin-H_2SO_4 and heating at 120°C, and observed under UV at 254 nm.

3.2. *Plant material*

The spines of *G. sinensis* Lam. were collected in October, 2003 in Guangxi in the southwest of China, which were identified by Prof. Tasi Liu of Hunan College of Traditional Chinese Medicine (Changsha, China). The spines were dried in the shade at room temperature to constant weight. A voucher specimen of the plant material was deposited in the Department of Plant Pathology, China Agricultural University, Beijing.

3.3. *Extraction and isolation*

The dry spines (12 kg) of *G. sinensis* were grounded into powder and then extracted twice with 95% ethanol (120 L) and 75% ethanol (120 L) under reflux at 80°C for 2 h. The ethanol extract solution was separated from the solid by filtration and evaporated to dryness under vacuum, yielding 696 g crude extract. The ethanol extract (600 g) was redissolved in 2 L hot water (60°C), and then extracted sequentially with petroleum ether (60–90°C), chloroform, ethyl acetate (EtOAc), and *n*-butanol (2 L each for three times). All solvent fractions and the remaining aqueous fraction were evaporated to dryness under vacuum, yielding 26.4 g petroleum ether fraction, 43.2 g chloroform fraction, 74.4 g EtOAc fraction, 130.8 g *n*-butanol fraction, and 390 g water fraction. The fractions were stored in a refrigerator at 4°C before use (for the antimicrobial activity tests or further fractionation). As the EtOAc fraction showed stronger inhibitory activity against *M. grisea*, it was selected for further isolation of antimicrobial compounds. The EtOAc fraction was fractionated in a silica gel column by gradient elution, starting with chloroform, and then various ratios of chloroform methanol. The fractions were examined with TLC, and those with a similar TLC pattern were combined, yielding ten fractions. Fraction III (4.40 g) showed antifungal activity and was further separated on a silica gel column, eluted with a

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gradient scheme of chloroform: methanol (1 : 0 to 0 : 1, v/v), yielding four distinct sub-fractions. Sub-fraction III-3 was further separated on a Sephadex LH-20 column, eluted with methanol to afford pure compound **1** (82 mg). Subfraction III-4 was further separated on a silica gel column, eluted with a gradient scheme of chloroform–methanol (4 : 1 to 0 : 1, v/v) to give pure compound **2** (70 mg).

3.4. *Acid hydrolysis*

According to the method described in the literature [13], a solution of 5 mg of **1** or **2** in 10 mL of MeOH–H_2O (1 : 1, v/v) mixed with 5 mL of 2N HCl was refluxed at 60°C for 6 h. The organic solvent was evaporated off under vacuum and the remaining aqueous phase was extracted three times with EtOAC. The EtOAC phase was removed and the remaining aqueous portion was concentrated and applied to TLC for the detection of arabinose for compound **1**, and rhamnose for compound **2**, by comparison with sugar standards.

3.5. *Antimicrobial tests*

The rice blast fungus *M. grisea*, obtained from the microbial culture stock in the Department of Plant Pathology, China Agricultural University, was used for the anti-fungal activity tests. The subculture and sporulation of *M. grisea* followed the documented procedures [15]. The antimicrobial activity of compounds was determined by the spore germination inhibition assay as described previously [16]. Amphotericin B (Amresco) was used as the positive control. Each treatment was repeated for five times. Spore germination was counted out of 100 spores under microscopy and percentage of spore germination was given by $[(G_c - G_t)/G_c] \times 100$, where G_c is the average germination count of the control, and G_t is the average germination count of the treatment. The median effective inhibitory concentration (IC_{50}) against spore germination of *M. grisea* was calculated by linear regression between the inhibitory probit and logarithmic drug concentration as described by Finney [17].

Acknowledgments

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