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A new epicatechin glucopyranoside derivative from *Styrax suberifolius*

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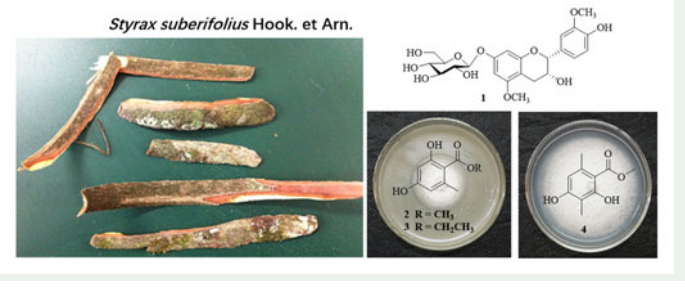
ABSTRACT

A new derivative of epicatechin glucopyranoside, (2*R*,3*R*)-3,7,4′-trihydroxy-5,3′-dimethoxyflavan 7-*O*-*β*-D-glucopyranoside (**1**), together with three mononuclear phenolic acid esters, methyl orsellinate (**2**), ethyl orsellinate (**3**) and methyl *β*-orcinolcarboxylate (**4**) were isolated from the bark of *Styrax suberifolius*. The structures of **1**–**4** were determined on the basis of extensive analysis of NMR and MS spectra combined with chemical hydrolysis. The antifungal activities of the isolated compounds against three plant pathogenic fungi, *Alternaria solani*, *Fusarium oxysporum* and *Phomopsis cytospore* were evaluated using radial growth inhibition assay. Compounds **2**, **3** and **4** exerted selective inhibitory activities against the tested fungi. Among of them, methyl *β*-orcinolcarboxylate (**4**) exhibited obvious inhibitory effect against *P. cytospore*, with an inhibition rate of 86.72% at 100 μg/ml.



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1. Introduction

Styrax Linn. is a genus of plants belonging to the family Styracaceae. The genus consists of more than 130 species mainly occurring in eastern Asia and southeastern America. *Styrax suberifolius* Hook, a tall tree, is distributed in south of the Yangtze

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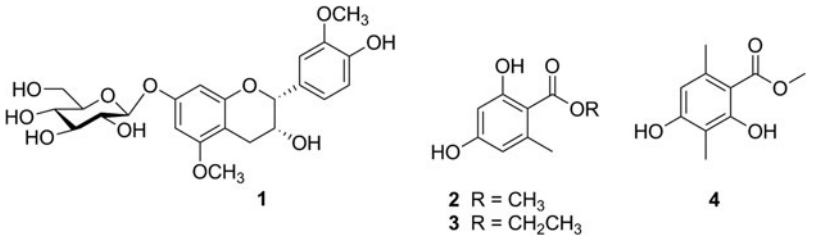

Figure 1. Chemical structures of compounds **1–4** isolated from *S. suberifolius*.

River in China and Vietnam that displays remarkable morphological variation throughout its range (Editorial Committee of Chinese flora 1987). Many species of *Styrax* have been used as traditional folk medicine worldwide (Chinese Pharmacopoeia Commission 2010) and about fifteen *Styrax* species have been phytochemically investigated (Liu et al. 2018). Previous phytochemical studies on this genus revealed various biologically active secondary metabolites including lignan derivatives, nor-neolignans, phenylpropanoids, phenolic acids, lipids, saponins and triterpenes (Pauletti et al. 2006; Bertanha et al. 2012; Timmers et al. 2015; Pazar and Akgül 2015; Scheuba et al. 2017). However, there have been very few studies about the chemical constituents of *S. suberifolius* in the literatures. Very recently, we have successfully isolated and identified seventeen phenylpropanoids from the bark of *S. suberifolius*, which represent the first phytochemical investigation of the species (Liu et al. 2018). In continuation of our phytochemical studies on this plant, herein, we report the isolation and structure identification of a new (–)-epicatechin glucopyranoside derivative (**1**) and three known mononuclear phenolic acid esters (**2–4**), as well as the antifungal activity of these compounds.

2. Results and discussion

The methanol extract of *S. suberifolius* was fractionated by a combination of silica gel column chromatography, Sephadex LH-20 column chromatography and preparative HPLC to yield (2*R*,3*R*)-3,7,4′-trihydroxy-5,3′-dimethoxyflavan 7-*O*-β-D-glucopyranoside (**1**), along with methyl orsellinate (**2**) (Cuellar et al. 2008), ethyl orsellinate (**3**) (Choudhary et al. 2011) and methyl β-orcinolcarboxylate (**4**) (Yusof et al. 2015).

Compound **1** was obtained as white solid powder with $[\alpha]_D^{25}$ + 9.9 (*c* 0.18, CH_3OH). The molecular formula was assigned as $C_{23}H_{28}O_{11}$ from its HR-ESIMS (*m/z* 515.1328 $[M + Cl]^-$) and NMR data. The ^{1}H- and ^{13}C-NMR spectra showed the presence of characteristic signals for a β-glucopyranose moiety and two methoxyls. Moreover, in the aromatic region two long-range coupling (^4J) proton signals at δ_H 6.37 (d, $J = 2.3$ Hz) and 6.39 (d, $J = 2.3$ Hz), as well as ABX-type proton signals at δ_H 6.92 (dd, $J = 2.0$, 8.1 Hz), 6.81 (d, $J = 8.1$ Hz) and 7.16 (d, $J = 2.0$ Hz) attributable to two aromatic rings were recognized. Besides, two oxygenated methine carbon signals at δ_C 80.1 (C-2) and 67.2 (C-3) and one methylene carbon signal at δ_C 29.5 (C-4) were observed in the ^{13}C-NMR spectrum. All above spectra data indicated that the structure of **1** could be a derivative of catechin or epicatechin glucopyranoside (Shen et al. 1993; Masika et al. 2004). With a careful interpretation of HSQC, ^{1}H-^{1}H COSY, HMBC and NOESY spectra,

Table 1. Antifungal effect of compounds**1**−**4** against *A. solani*, *F. oxysporum* and *P. cytospore* at 100 μg/ml.

	Colony diameter[a] (mm)			Inhibition[b] (%)		
Compound (number)	*A. solani*	*F. oxysporum*	*P. cytospore*	*A. solani*	*F. oxysporum*	*P. cytospore*
1	>55	>55	>50	—	—	—
2	30.0 ± 0.94	25.0 ± 0.82	>50	58.41	67.39	—
3	29.5 ± 0.62	37.0 ± 0.41	>50	59.31	45.65	—
4	>55	28.3 ± 0.24	13.2 ± 0.24	—	61.41	86.72
Control	62.3 ± 0.43	62.2 ± 2.13	53.7 ± 2.25	0	0	0

[a]Mean values of three replicated of colony diameter ± S.D.
[b]Percentage of colony growth inhibition respective to the control (contained solvent without the compounds).

the planar structure of the aglycone was elucidated to be 3,7,4′-trihydroxy-5,3′-dimethoxyflavan, while the small diagnostic coupling values between H-2 and H-3 ($J_{2,3}$ < 1 Hz) favored these protons being in a epicatechin-type arrangement (Morimoto et al. 1985). Furthermore the β-glucopyranosyl was deduced to be bound at C-7 via a glycosidic linkage from long-range correlations of H-1″ [δ 4.88 (d, J = 7.8 Hz)] with C-7 (δ 158.9) observed in the HMBC spectrum. Acid hydrolysis of **1** liberated D-glucose along with aglycone (**1a**). The aglycone was identified as 5,3′-di-*O*-methyl-(−)-epicatechin which was confirmed by comparison of the NMR data and optical rotation value (Joo et al. 2014) with those reported in the literatures. Compound **1** was finally characterized as (2*R*,3*R*)-3,7,4′-trihydroxy-5,3′-dimethoxyflavan 7-*O*-β-D-glucopyranoside (Figure 1). It should be noted that a similar structure named Glochiflavanoside A has already been isolated from *Glochidion zeylanicum* (Otsuka et al. 2001), but in that case, the aglycone was (2*R*,3*S*)-3,7,4′-trihydroxy-5,3′-dimethoxyflavan.

In the course of this research, it was noticed that CH_3OH-soluble extracts of *S. suberifolius* are resistant to plant pathogenic fungi. The antifungal activities of **1**−**4** against *A. solani*, *F. oxysporum* and *P. cytospore* were evaluated using radial growth inhibition assay at 100 μg/ml. As shown in Table 1, compound **1** has no inhibitory effect against the three fungi, but compounds **2**, **3** and **4** exerted selective inhibitory activities. Among which, compounds **2** and **3** showed moderate activity against *A. solani* and *F. oxysporum*, while **4** against *F. oxysporum* and *P. cytospore*. Methyl β-orcinolcarboxylate (**4**) exhibited obvious inhibitory effect against *P. cytospore* with an inhibition rate of 86.72%. It is noteworthy that the compounds **2**−**4** are a class of antimicrobial mononuclear phenolic acid esters, which are frequently isolated from lichen species (Molnár and Farkas 2010). Previous reports have demonstrated that methyl orsellinate (**2**) and methyl-β-orcinolcarboxylate (**4**) showed significant antifungal activity against *Trichophyton longifusis, Microsporum canis, Aspergillus flavus* and *Fusarium solani* at a concentration of 200 μg/ml (Thadhani et al. 2012). The present study extends the antifungal activity of metabolites **2**−**4** to plant pathogenic fungi *A. solani*, *F. oxysporum* and *P. cytospore*.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined using a Perkin-Elmer 341 polarimeter (PerkinElmer Co., Waltham, MA, USA). HRESIMS spectra were taken on an API QSTAR mass

spectrometer (Applied Biosystem/MSD Sciex, Concord, ON, Canada). The UV-VIS absorbance were measured with a Shimadzu UV-1800 spectrophotometer (Shimadzu Pte., Ltd., Singapore). CD spectra were recorded with a J-810 spectrophotometer (JASCO Co., Japan). The ^{1}H, ^{13}C, and 2D NMR spectra were recorded on a Bruker Avance III 600 MHz NMR spectrometer using TMS as an internal standard. Column chromatography was performed on silica gel 60 (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China). Semi-preparative HPLC was performed on a Waters 1525 Binary HPLC pump and a Waters 2414 refractive index detector using a YMC-Pack ODS-A column (250 mm × 10 mm I.D.; S-5 μm, 12 nm). Analytical TLC plates (HSGF254, Jiangyou silicone Development Co., Ltd., Yantai, China), Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) and Develosil ODS (50 μm, Nomura Chemical Co. Ltd., Osaka, Japan) were used for the isolation experiments.

3.2. Plant material

Bark from trunks of *S. suberifolius* were collected in western Hunan province, China, in December 2012, which identified by Prof. Dai-gui Zhang (Key laboratory of Plant Resources Conservation and Utilization, Jishou University). A voucher specimen (zdg20121214) was deposited in Hunan Agricultural University.

3.3. Extraction and isolation

Dried bark of *S. suberifolius* (3.0 kg) was powdered and extracted with CH_3OH (10 L, 24 h × 3) at room temperature and the organic solvent was concentrated under vacuum to yield a crude extract (180.0 g). The CH_3OH-soluble extract was directly subjected to a silica gel column chromatography (CC), eluting with petroleum ether-acetone (9:1→7:3, v/v) and $CHCl_3$- CH_3OH (9:1→6:4, v/v), to obtain six fractions (Fr.A – Fr.F). Fraction Fr.B (0.9 g) was decolorized over MCI gel (eluted with 90% CH_3OH) and then was separated on an ODS-C18 column eluting with acetone-H_2O (5:5→10:0, v/v), to afford six sub-fractions (Fr.B1 – Fr.B6). Sub-fraction B2 with clear spots monitored by TLC, was further purified by Sephadex LH-20 column (eluted with $CHCl_3$-CH_3OH, 1:1) to afford compound **4** (54.0 mg). Compounds **3** (19.5 mg, t_R = 15 min) and **2** (20.0 mg, t_R = 18 min) were obtained from sub-fraction B4 which purified on Sephadex LH-20 column and semi-preparative HPLC chromatography (elution solution: 90% CH_3OH-H_2O, flow rate: 3 ml/min). Similarly, Fr.E (2.0 g) was successively separated by ODS-C18 column eluting with CH_3OH-H_2O (3:7→7:3, v/v) and Sephadex LH-20 column (eluted with CH_3OH). Finally, compound **1** (24.0 mg) was yielded from sub-fraction E2 using semi-preparative HPLC chromatography (65% CH_3OH-H_2O, flow rate 3 ml/min, t_R = 15.0 min).

Compound **1**: White solid; $[\alpha]_D^{25}$ + 9.9 (*c* 0.18, CH_3OH); UV (CH_3OH): λ_{max} 205 (2.23), 227sh (0.51), 278 (0.09) nm (AU); CD Δε (nm): +8.56 (209), +2.22 (228), +0.51 (283) ($c = 1.87 \times 10^{-3}$ M, MeOH); positive ion ESIMS *m/z*: 481 $[M + H]^+$, 503 $[M + Na]^+$; negative ESIMS *m/z*: 479 $[M - H]^-$, 515 $[M + Cl]^-$; HR-ESIMS *m/z*: 515.1328 $[M + Cl]^-$ (calcd for $C_{23}H_{28}O_{11}Cl$,515.1325). ^{1}H NMR (CD_3OD, 600 MHz), δ 7.16 (1H, d, $J = 2.0$ Hz, H-2′), 6.92 (1H, dd, $J = 2.0$, 8.1 Hz, H-6′), 6.81 (1H, d, $J = 8.1$ Hz, H-5′), 6.39 (1H, d, $J = 2.3$ Hz, H-

8), 6.37 (1H, d, $J = 2.3$ Hz, H-6), 4.91 (1H, br s, H-2), 4.22 (1H, m, H-3), 3.88 (3H, s, 3′-OMe), 3.82 (3H, s, 5-OMe), 2.92 (1H, dd, $J = 4.6$, 17.1 Hz, H-4β), 2.81 (1H, dd, $J = 2.3$, 17.1 Hz, H-4*a*), 4.88 (1H, d, $J = 7.8$ Hz, H-1″), 3.46 (1H, m, H-2″), 3.44 (1H, m, H-3″), 3.38 (1H, dd, $J = 8.5$, 9.9 Hz, H-4″), 3.46 (1H, m, H-5″), 3.70 (1H, dd, $J = 6.0$, 12.1 Hz, H-6″a), 3.91 (1H, dd, $J = 2.3$, 12.1 Hz, H-6″b); ^{13}C NMR (CD_3OD, 150 MHz), δ 80.1 (C-2), 67.2 (C-3), 29.5 (C-4), 160.4 (C-5), 98.4 (C-6), 158.9 (C-7), 94.5 (C-8), 157.0 (C-9), 103.9 (C-10), 132.0 (C-1′), 111.9 (C-2′), 148.6 (C-3′), 147.1 (C-4′), 115.7 (C-5′), 120.6 (C-6′), 102.7 (C-1″), 74.9 (C-2″), 78.2 (C-3″), 71.5 (C-4″), 78.0 (C-5″), 62.6 (C-6″), 56.0 (5-OMe), 56.4 (3′-OMe).

3.4. Acid hydrolysis of 1 and identification of its aglycone and D-Glucose

Compound **1** (11.0 mg) in 50 ml of 1 M HCl (H_2O/1,4-dioxane, 1:1) was heated under reflux for 6 h. After removal of the solvent, the residue was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble portion was evaporated and subjected to ODS column chromatography using 90% CH_3OH to obtain the aglycone **1a** (4.5 mg). Aglycone **(1a)** gave as a yellow powder, its optical rotation $[\alpha]_D^{25}$ −69.9 (*c* 0.2, CH_3OH) as well as ^{1}H and ^{13}C NMR (CD_3OD, 600 MHz) data are in accordance with those reported in the previous papers (Joo et al. 2014).

The water layer was neutralized with 5% NaOH and desalted using a Sephadex LH-20 column (CH_3OH) to afford a sugar residue (3.5 mg). The sugar was confirmed to be D-glucose by comparison with an authentic sample on TLC [Merck Kieselgel 60GF254, EtOAc-CH_3OH-H_2O-AcOH (6.5:2.0:1.5:1.5), R*f* = 0.40] and by measurement of its optical rotation ($[\alpha]_D^{25}$ + 71.0, *c* 0.35, H_2O).

3.5. Antifungal assay

Alternaria solani, *Fusarium oxysporum* and *Phomopsis cytospore* were obtained from Hunan Provincial Key Laboratory for Biology and Control of Plant Diseases and Insect Pests. The three fungi were incubated on a slant that had 10 ml of a potato dextrose agar (PDA) medium for 7 days at 28 °C in dark.

The effects of compounds **1 − 4** on colony growth of the three plant pathogenic fungi were evaluated according to the procedure described by Vargas-Arispuro et al. (2005). Pure compounds were dissolved in 500 µl of acetone and 10 µl of Twain-80 was added as emulsifier. Then diluted with sterile water to 5 ml. These solutions were incorporated into 5 ml of PDA media at final concentrations of 100 µg/ml. Three plates of solid PDA media containing the compounds at the specified concentrations were centrally point-inoculated with mushroom cakes from 7-day-old cultures of *A. solani*, *F. oxysporum* and *P. cytospore*. The control plates were included as blanks that contained solvent without the compounds. Cultures were incubated in the dark at 28 °C. The percentage of growth inhibition was obtained by comparing the experimental with the control, which was considered to be 100% growth. Experimental set was run in triplicate, and the mean colony diameter and standard deviation (S.D.) of the mean values were calculated.

4. Conclusions

In this study, we identified a new derivative of epicatechin glucopyranoside and three known mononuclear phenolic acid esters in the bark of *S. suberifolius*. Methyl orsellinate (**2**), ethyl orsellinate (**3**) and methyl β-orcinolcarboxylate (**4**) have selective inhibitory activities against plant pathogenic fungi. Compound **4** exhibited obvious inhibitory effect against *P. cytospore* at 100 μg/ml, with an inhibition rate of 86.72%. Therefore, these compounds may have a biopesticidal potential as control agents for plant pathogenic fungi.

Disclosure statement

No potential conflict of interest was reported by the authors.

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