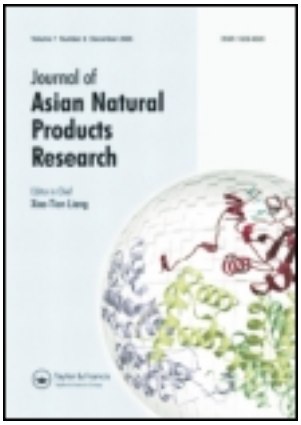

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Antiviral dicaffeoyl derivatives from *Elephantopus scaber*

Hua-Wei Geng[ab†], Xiao-Li Zhang[ab†], Guo-Cai Wang[ab], Xin-Xin Yang[ab], Xia Wu[ab], Yi-Fei Wang[c], Wen-Cai Ye[ab] and Yao-Lan Li[ab]*

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A new dicaffeoyl derivative, 1α,2β-*O*-dicaffeoylcyclopentan-3β-ol (**1**), along with four dicaffeoylquinic acids (**2**–**5**), was isolated from the rhizomes of *Elephantopus scaber* Linn. The structure of the new compound was elucidated on the basis of extensive spectroscopic analyses. The *in vitro* antiviral activity against respiratory syncytial virus (RSV) of these dicaffeoyl derivatives was evaluated by cytopathic effect reduction assay, and all of them displayed more potent anti-RSV activity than ribavirin, the positive control drug.

Keywords: *Elephantopus scaber*; dicaffeoyl derivatives; antiviral; respiratory syncytial virus

1. Introduction

Elephantopus scaber Linn. (Compositae) is a medicinal herb traditionally used in many countries and regions to stimulate diuresis, reduce fever, and eliminate bladder stones, as well as to treat nephritis, edema, dampness, chest pain, pneumonia, scabies, and arthralgia [1]. The rhizome of *E. scaber*, known as 'di dan tou' in southern China, is commonly used to treat common cold, pertussis, and inflammations [2]. Recent researches have proved that *E. scaber* has anticancer, antibacterial, anti-inflammatory, hepatoprotective, and diuretic effects; its chemical constituents mainly include sesquiterpene lactones, triterpenes, flavonoids, and caffeoylquinic acids [2]. Our previous studies indicated that the aqueous extract of *E. scaber* possessed *in vitro* anti-respiratory syncytial virus (RSV) activity with 50% inhibition concentration (IC_{50}) value of 23.5 μg ml^{-1} and selectivity index (SI) value of 11.3, and its active components may be polyphenols [3]. Therefore, a bioassay-guided isolation of *E. scaber* was undertaken in the present study, leading to obtain five dicaffeoyl derivatives including a new compound, 1α,2β-*O*-dicaffeoylcyclopentan-3β-ol (**1**) (Figure 1), along with four dicaffeoylquinic acids (**2**–**5**). Bioassay showed that all of the dicaffeoyl derivatives possessed significant *in vitro* anti-RSV activity. This article deals with the isolation, structural elucidation, and antiviral evaluation of these dicaffeoyl derivatives.

2. Results and discussion

The dried and powdered rhizomes of *E. scaber* were extracted with 95% ethanol and then partitioned with petroleum ether,

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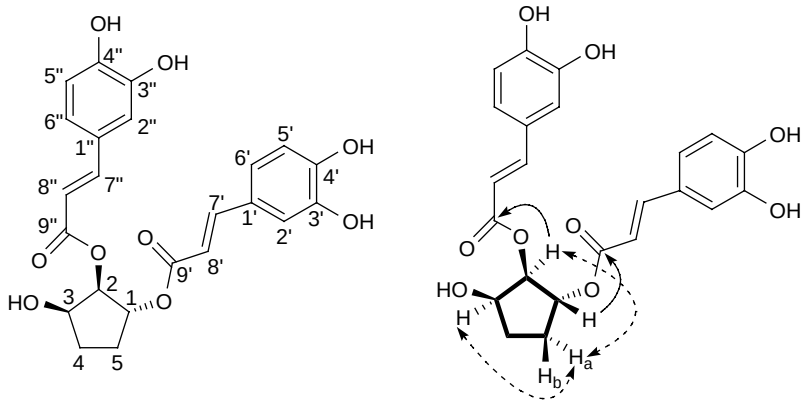

Figure 1. The chemical structure, key HMBC (HC), COSY (H–H), and ROESY (HH) correlations of **1**.

ethyl acetate, and *n*-butanol, respectively, to obtain three fractions. Owing to the potent anti-RSV activity, the *n*-butanol fraction was subjected to repeated column chromatography over Diaion HP-20, Sephadex LH-20, and preparative HPLC to afford compounds **1**–**5**. Four known compounds were identified as 4,5-di-*O*-caffeoyl quinic acid (**2**) [4], 3,4-di-*O*-caffeoyl quinic acid (**3**) [4], 4,5-di-*O*-caffeoyl quinic acid methyl ester (**4**) [5], and 3,4-di-*O*-caffeoyl quinic acid methyl ester (**5**) [5] by comparing their physical and spectroscopic data with those reported in the literature. Compounds **3**–**5** were isolated from the herb for the first time.

Compound **1** was obtained as an amorphous yellowish powder with $[\alpha]_D^{24}$ −252 ($c = 0.50$, MeOH). Its HR-ESI-MS showed an $[M + Na]^+$ ion at *m/z* 465.1156, corresponding to the molecular formula of $C_{23}H_{22}O_9$. The UV spectrum of **1** exhibited the absorption maxima at 217, 243, 300, and 326 nm, indicating the presence of caffeoyl group [6]. The IR spectrum of **1** showed the presence of hydroxyl (3383 cm^{-1}), carbonyl (1684 cm^{-1}), and phenyl (1597 and 1521 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) showed four olefinic protons of two *trans* double bonds at δ_H 7.59, 6.27, 7.50, and 6.18 (each 1H, d, $J = 16.0$ Hz), six aromatic protons at δ_H 7.00 and 6.98 (each 1H, d, $J = 2.0$ Hz), 6.72 (2H, d, $J = 8.0$ Hz) and 6.87 (2H, dd, $J = 8.0, 2.0$ Hz), and three proton signals of oxymethines at δ_H 5.65 (1H, m), 5.12 (1H, dd, $J = 8.3, 2.8$ Hz), and 4.37 (1H, m). The ^{13}C NMR spectrum of **1**

Table 1. ^1H and ^{13}C NMR spectral data of **1** (δ in ppm, J in Hz, in MeOD).

Position	^{13}C	^1H
1	69.8	5.65 m
2	76.2	5.12 dd (8.3, 2.8)
3	69.1	4.37 m
4	38.5	2.29[a]
5	39.5	2.13 m
	–	2.27[a]
1′	127.7	–
2′	114.7	7.00 d (2.0)
3′	146.7	–
4′	149.6	–
5′	116.5	6.72 d (8.0)
6′	123.1	6.87 dd (8.0, 2.0)
7′	147.7	7.59 d (16.0)
8′	115.2	6.27 d (16.0)
9′	168.6	–
1″	127.6	–
2″	114.7	6.98 d (2.0)
3″	146.7	–
4″	149.6	–
5″	116.5	6.72 d (8.0)
6″	123.1	6.87 dd (8.0, 2.0)
7″	147.5	7.50 d (16.0)
8″	115.2	6.18 d (16.0)
9″	168.4	–

Notes: 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. The carbon and proton signals were assigned by ^1H–^1H COSY, HSQC, and HMBC spectroscopic data.

[a] Overlapped signals were reported without designating multiplicity.

(Table 1) showed the carbon signals of two carbonyls (δ_C 168.4, 168.6), two double bonds (δ_C 147.7, 147.5, 115.2, 115.2), three oxymethines (δ_C 76.2, 69.8, 69.1), and two phenyls. The above data suggested the presence of two caffeoyl groups [7]. The spin system of protons at δ_H 5.65 (H-1) ↔ 5.12 (H-2) ↔ 4.37 (H-3) ↔ 2.29 (H-4) ↔ 2.13 (H-5) ↔ 5.65 (H-1) (Figure 1) was displayed by the $^1H-^1H$ COSY spectrum, indicating the presence of one 1,2,3-cyclopentanetriol moiety. The HMBC correlations between H-1 at δ_H 5.65 and C-9′ at δ_C 168.6, and between H-2 at δ_H 5.12 and C-9″ at δ_C 168.4 (Figure 1), documented that the two caffeoyl groups were connected to the C-1 and C-2 positions, respectively. The relative configuration of **1** was assigned by the NOESY correlations of H-2 (δ_H 5.12)/H-5a (δ_H 2.13) and H-3 (δ_H 4.37)/H-5a (δ_H 2.13), and no cross peak of H-1 (δ_H 5.65)/H-3 (δ_H 4.37) (Figure 1). So the structure of **1** was elucidated as 1α,2β-*O*-dicaffeoylcyclopentan-3β-ol.

All the isolated compounds were tested for their *in vitro* antiviral activities against RSV using cytopathic effect (CPE) reduction assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method was applied to measure the cytotoxicity of the compounds. The SI value calculated from the ratio of CC_{50}/IC_{50} was used as an important parameter to evaluate the antiviral activity of the compounds. As shown in Table 2, five of the dicaffeoyl derivatives possessed strong anti-RSV activity with IC_{50} values lower than that of ribavirin, and compounds **1**–**3** showed lower cytotoxicity to HEp-2 cells, resulting in the higher SI values. Of these dicaffeoyl derivatives, the new compound (**1**) was most active with the highest SI value. Currently, quite a few studies concern the antiviral activities of the dicaffeoyl derivatives, i.e. the dicaffeoylquinic acids against RSV [6], as well as dicaffeoylquinic acids and dicaffeoyltartaric acids against human immunodeficiency virus type 1 (HIV-1) [8]. Our study firstly revealed the anti-RSV activity of a dicaffeoylcyclopentanol derivative (**1**). It is noteworthy that although 1α,2β-*O*-dicaffeoylcyclopentan-3β-ol (**1**) lacks the carboxyl group(s) in its linker moiety (cyclopentanetriol) of two caffeoyl units as compared with dicaffeoylquinic acids and dicaffeoyltartaric acids, its antiviral activity remains strong.

3. Experimental

3.1 General experimental procedures

Optical rotations were carried out using a Jasco P-1020 digital polarimeter. The IR spectra were determined on a JASCO FT-IR-480 plus infrared spectrometer as KBr pellets. The ESI-MS spectra were measured by a Finnigan LCQ Advantage MAX ion trap mass spectrometer. The NMR

Table 2. Anti-RSV activity of the dicaffeoyl derivatives from *E. scaber*.

Compounds	IC_{50} ($\mu g\,ml^{-1}$)[a]	CC_{50} ($\mu g\,ml^{-1}$)[b]	SI	IC_{100} ($\mu g\,ml^{-1}$)[c]	MNCC ($\mu g\,ml^{-1}$)[d]
1	0.63	>200	>318.65	5.00	100.00
2	1.25	>200	>160	6.25	50.00
3	1.50	>200	>133.33	10.00	50.00
4	0.63	118.68	99.73	5.00	20.00
5	0.78	75.90	97.42	5.00	12.50
Ribavirin[e]	1.50	62.50	41.67	> 6.25	6.25

Notes: [a] IC_{50} is the concentration that reduced 50% of CPE with respect to virus control.
[b] CC_{50} is the concentration of sample with half maximal inhibition on the growth and survival of HEp-2 cells.
[c] IC_{100} is the concentration that reduced 100% of CPE with respect to virus control.
[d] MNCC is the maximal nontoxic concentration of sample.
[e] Ribavirin is the positive control drug.

spectra were recorded on a Bruker-AV-400 spectrometer. The HR-ESI-MS spectra were recorded on an Agilent 6210 LC/MSD TOF mass spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), macroporous resin (Diaion HP-20, Shanghai, China), Sephadex LH-20 (25–100 μm, Fluka, Buchs, Switzerland), octadecylsilyl (ODS) (50 μm, YMC, Tokyo, Japan), and preparative HPLC were used for column chromatography. Preparative HPLC was performed using a Varian liquid chromatograph equipped with a Waters $5C_{18}$-MS-II column (20 mm ID × 15 cm). Fractions were monitored by $HSGF_{254}$ silica gel TLC plates (0.2 mm thickness, 10 × 20 cm, Qingdao Marine Chemical Inc., Qingdao, China).

3.2 Plant material

The rhizomes of *E. scaber* were collected from Baiyun Mountain, Guangzhou, China, and were authenticated by Mr Zhen-Qiu Mai, a senior herbalist at the Chinese Medicinal Material Company, Guangzhou, China. Voucher specimen was deposited at the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University with an accession number of 06111207.

3.3 Extraction and isolation

The dried and powdered rhizomes of *E. scaber* (20.0 kg) were soaked in 95% ethanol and extracted by percolating. The ethanol solution was then concentrated *in vacuo* to yield a crude extract (1.38 kg). The crude extract (1.3 kg) was suspended in distilled water and partitioned successively with petroleum ether, ethyl acetate, and *n*-butanol. After the evaporation under reduced pressure, the petroleum ether fraction (600 g), ethyl acetate fraction (230 g), and *n*-butanol fraction (200 g) were obtained, respectively, and then were subjected to the antiviral evaluation using CPE reduction assay. The *n*-butanol fraction was screened out to further separate due to its potent anti-RSV effect. The *n*-butanol fraction (190 g) was fractionated on a Diaion HP-20 column eluted successively by water, 30% ethanol, 60% ethanol, and 95% ethanol to afford four fractions. The 60% ethanol eluting fraction, possessing potent anti-RSV effect, was further isolated on a Sephadex LH-20 column eluted using $CHCl_3$–MeOH (1:1) to afford two subfractions. Subfraction 1 (1.2 g) was separated by preparative HPLC column eluted using 50% methanol to yield compounds **1** (10.3 mg), **2** (12.5 mg), and **3** (8.3 mg), and subfraction 2 (2.0 g) was separated by preparative HPLC column eluting with 60% methanol to yield compounds **4** (22.0 mg) and **5** (9.6 mg), respectively.

3.3.1 1α,2β-O-Dicaffeoylcyclopentan-3β-ol (1)

Amorphous yellowish powders, $[\alpha]_D^{24} - 252$ ($c = 0.50$, MeOH). UV (MeOH) λ_{max} (log ε): 217, 243, 300, and 326 nm. IR (KBr) ν_{max}: 3383, 1684, 1597, 1521, and 1272 cm^{-1}. For ^1H NMR (400 MHz, MeOD) and ^{13}C NMR (100 MHz, MeOD) spectral data, see Table 1. ESI-MS (positive): *m/z* 443 $[M + H]^+$ and 465 $[M + Na]^+$. HR-ESI-MS (positive): *m/z* 465.1156 $[M + Na]^+$ (calcd for $C_{23}H_{22}O_9Na$, 465.1152).

3.4 Antiviral activity

3.4.1 Cell and virus

Human larynx epidermoid carcinoma cell line (HEp-2 cells) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RSV (long strain) was obtained from Medical Virology Institute, Wuhan University. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 25 $\mu g\,ml^{-1}$ gentamicin, and 200 mM L-glutamine (growth medium). Virus-infected cells were maintained in EMEM with 2% FBS, 25 $\mu g\,ml^{-1}$ gentamicin, and 200 mM L-glutamine (maintenance medium). All the

cells were cultured at 37°C in a humidified atmosphere supplied with 5% CO_2. Virus titers were determined by the 50% tissue culture infective dose ($TCID_{50}$) method.

3.4.2 Cytotoxicity assay

The cytotoxicity of the samples was tested by the MTT method as described in the previous study [9]. Briefly, 100 μl of twofold diluted samples was added to a 96-well plate containing confluent cell monolayer in triplicates, while the dilution medium without the sample was the control. After 72 h of incubation, 10 μl of the MTT solution (5 mg ml^{-1} in phosphate buffered saline) was added to each well. The trays were further incubated for 4 h for the formation of blue formazan. After the supernatant was removed, the blue formazan was solubilized in 100 μl DMSO and the optical density was measured at double wavelength of 570 and 690 nm with a microplate reader.

3.4.3 Antiviral assay

The CPE reduction assay was adopted to evaluate antiviral activity of the samples as described in the previous study [9]. Briefly, 0.1 ml of 100 $TCID_{50}$ virus suspension and serial twofold dilutions of the tested samples were added simultaneously to confluent cell monolayer in a 96-well plate. Virus suspension and maintenance medium without samples were added as the virus control and cell control, respectively. The plates were incubated at 37°C in a humidified CO_2 atmosphere for 4–5 days. The virus-induced CPE in each well was observed against the virus control under a light microscope. Ribavirin (Sigma, St. Louis, MO, USA) was used as positive control in this experiment.

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