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小叶榕叶中具有抗 HSV 活性的黄烷成分

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摘要: 从小叶榕(*Ficus microcarpa* L.)叶的乙醇提取物中首次分离鉴定了(+)(2R,3S)阿夫儿茶素(**1**)、(-)(2R,3R)表阿夫儿茶素(**2**)、(-)(2R,3S)阿夫儿茶素-(4α-8)(2R,3S)阿夫儿茶素(**3**)、(-)(2R,3S)阿夫儿茶素-(4α-8)(2R,3R)表阿夫儿茶素(**4**) 4 个黄烷化合物,其中化合物 **1** 和 **2** 对兔肾细胞中培养的 2 型单纯疱疹病毒(HSV-2)具有抑制作用,半数抑制浓度(IC_{50})分别为 0.49 和 0.55 mg mL^{-1},治疗指数(TI)分别为 4.31 和 3.19,可用作小叶榕叶药材质量控制的指标成分。从该提取物中还分离鉴定了另外 7 个已知成分:儿茶素(**5**)、phaseic acid (**6**)、苯甲酸(**7**)、4-羟基苯甲酸(**8**)、间苯三酚(**9**)、胡萝卜苷(**10**)和 β-谷甾醇(**11**)。

关键词: 小叶榕; 叶; 黄烷; 阿夫儿茶素; 表阿夫儿茶素; 单纯疱疹病毒
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Flavans with Anti-HSV Activity from the Leaves of *Ficus microcarpa* L.

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Abstract: Four flavans, named as (+)(2R,3S) afzelechin (**1**), (-)(2R,3R) epiafzelechin (**2**), (-)(2R,3S)afzelechin-(4α-8)(2R,3S) afzelechin (**3**) and (-)(2R,3S) afzelechin-(4α-8) (2R,3R)epiafzelechin (**4**), were isolated from ethanolic extract of the leaves of *Ficus microcarpa* L. for the first time. These compounds were evaluated antiviral effects *in vitro* towards herpes simplex virus (HSV). Flavans **1** and **2** exhibited low toxicity to rabbit kidney primary cells, moderate antiviral activity against HSV-1 with IC_{50} values of 0.49 and 0.55 mg mL^{-1}, and therapeutic indexes (TI) of 4.31 and 3.19, respectively. According to cytotoxity and cytopathic effect (CPE) assays, they can be recommended as reference markers for quality control of *Folium Fici microcarpae*. Other seven known compounds, such as (+) catechin (**5**), phaseic acid (**6**), benzoic acid (**7**), 4-hydroxy-benzoic acid (**8**), phloroglucinol (**9**), daucosterol (**10**), and β-sitosterol (**11**), were also identified.

Key words: *Ficus microcarpa* L.; Leaf; Flavan; Afzelechin; Epiafzelechin; HSV

Ficus microcarpa L. f. (Moraceae) is a kind of arbor widely distributed in the tropical and subtropical areas of China[1]. *Folium Fici microcarpae,* the dried leaves of this plant, is a Chinese materia medica used for chronic bronchitis, flu, tonsillitis, and is used as a chief component in a Chinese medicine formulation '*Ke-Te-Ling*' for cough treatment[2]. Previously, triterpenoids have been isolated from the aerial roots of this plant[3], however, only a few bioactive constituents of the leaves were reported[4]. The compounds isolated from the leaves of *F. microcarpa* and *in vitro* antiviral effects of some flavans were reported in this paper (Fig. 1).

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1 Materials and methods

1.1 Plant constituents

The leaves of *Ficus microcarpa* L. were collected in Guangzhou, China and identified by Prof. CHEN Wen-feng of South China Botanical Garden, Chinese Academy of Sciences. Compounds **1** ~ **11** were isolated from the leaves of *F. microcarpa* by means of chromatography. Briefly, the air-dried leaves (5.0 kg) were powdered and extracted with EtOH at room temperature. The ethanol extract was extracted sequentially with petroleum ether and EtOAc. The EtOAc extract (60 g) was chromatographed repeatedly on silica gel and ODS columns, eluted with $CHCl_3$/MeOH (20: 1 to 8: 1) and MeOH/H_2O (1: 9 to 8: 2) to obtain compounds **1** (15 mg), **2** (20 mg), **3** (20 mg), **4** (18 mg), **5** (10 mg), **6** (15 mg), **7** (100 mg), **8** (20 mg), **9** (10 mg), **10** (40 mg), and **11** (15 mg), respectively. Their melting points were determined with a SGW X-4 micromelting point meter, optical rotations were measured on a WZZ-IS polarimeter, ESI-MS or HR-FABMS were measured on an API 2000 LC/MS/MS apparatus or a MAT95XP mass spectrometer, ^1H and ^{13}C NMR were recorded on a Bruker DRX-400 instrument with TMS as internal standard.

1.2 Cell, virus and test compound

Rabbit kidney primary cells were isolated from the one-day-old New Zealand rabbit as described[5]. Herpes simplex viruses type 1 (VR733 F strain of HSV-1, from ATCC) and type 2 (strain333 of HSV-2, from the Virology Institute of Medical School, Wuhan University, China) were cultured in the rabbit kidney primary cells at 37℃ in a humidified atmosphere with 5% CO_2 and 2% DEME medium supplemented with 10% fetal bovine serum (FBS). The infectivity of viruses was expressed as $TCID_{50}$ (50% tissue culture infection dose). Compounds **1** ~ **4** were dissolved in DMSO, diluted with 2% DEME medium, and adjusted to pH 7.

1.3 Cytotoxicity assay

100 μL of the rabbit kidney primary cells suspension, containing 1.5×10^5 cells, was planted into each well of 96-well microplates and incubated in 2% DMEM (Gibco) medium supplementing with 10% FBS, at 37℃ in 5% CO_2 for 24 hours. The medium was removed and a fresh medium, containing different concentration (20 ~ 6600 μg mL^{-1}) of test compounds, or positive control, acyclovir (ACV, Livzon Pharmaceutical Co., Ltd, China), was added in quadruplicate. After incubation for 4 days, cytotoxicity was evaluated and compared to cell control using MTT assay[5]. Solvent (DMSO) control and blank were also included. The concentration which reduced viability of cells to 50% of the cell control was expressed as the 50% cytotoxic concentration (CC_{50}).

1.4 Cytopathic effect (CPE) reduction assay

Antiviral activities of compounds **1** ~ **4** against HSV-1 and HSV-2 were tested using a CPE reduction assay. Briefly, the rabbit kidney primary cells were seeded in 96-well microplates and grew to a uniform monolayer under the conditions described above. Compounds of different concentrations were added in quadruplicate, immediately followed by the addition of 100 $TCID_{50}$ of virus in 50 μL maintenance medium. The plates were incubated at 37℃ in 5% CO_2. For each concentration, 4 wells remained uninfected to observe the morphological change due to the cytotoxicity of the testing compound. Growth of the cells infected with the viruses HSV-1 and HSV-2 treated with the compounds was compared with control cells, which were infected with virus only. Acyclovir and DMSO were used as positive and solvent control, respectively. The compounds were tested at non-cytotoxic concentrations (> 50% host cell grow) and concentration of DMSO in the media was maintained at less than 0.63% to ensure that it did not affect the growth of cells. The CPE was examined and counted under an inverted microscope (100 ×) everyday and graded as no CPE in the cells infected by virus (marked -), or gradual CPE in cells infected by virus [marked + (0 ~ 25%), 2 + (25% ~ 25%), 3 + (50% ~ 75%), and 4 + (75% ~ 100%), respectively]. The concentration of the antiviral sample reducing CPE by 50% (IC_{50}) is determined

from the concentration response curve, and therapeutic index (TI) is calculated from the ratio of CC_{50}/IC_{50}.

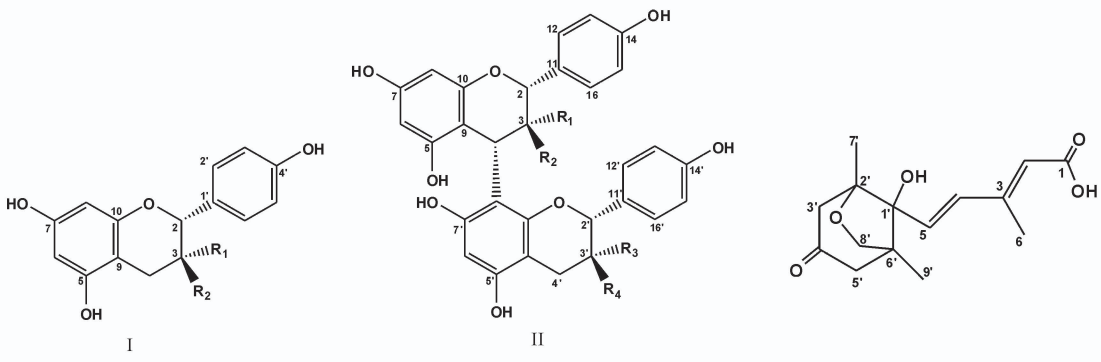

Fig. 1 Structures of compounds **1~6** from the leaves of *F. microcarpa*

2 Results and discussion

2.1 Structural identification

Chemical structures of the eleven compounds were elucidated on the basis of spectroscopic and chemical evidences as (+)(2R,3S) afzelechin (**1**), (-)(2R,3R) epiafzelechin (**2**), (-)(2R,3S) afzelechin-(4α-8)(2R,3S) afzelechin (**3**), (-)(2R,3S)afzelechin-(4α-8)(2R,3R) epiafzelechin (**4**), (+)catechin (**5**), phaseic acid (**6**), benzoic acid (**7**), 4-hydroxy-benzoic acid (**8**), phloroglucinol (**9**), daucosterol (**10**), and β-sitosterol (**11**). Flavans **1**~**4** were found from *Ficus microcarpa* L. for the first time.

(+)(2R,3S)Afzelechin (1)[6-7] Yellow crystals; mp 220 ~222℃, $[\alpha]_D^{20}$ 6.17° (*c* 0.32, acetone); ^{1}H NMR (acetone-d_6, 400 MHz) δ (ppm): 4.57 (1H, d, *J* = 8.4 Hz, H-2β), 3.97 (1H, ddd, *J* = 5.6, 8.4, 8.4 Hz, H-3α), 2.51 (1H, dd, *J* = 5.6, 16.0 Hz, H-4α), 2.91 (1H, ddd, *J* = 8.4, 8.4, 16.0 Hz, H-4β), 6.01 (1H, d, *J* = 2.0 Hz, H-6), 5.85 (1H, d, *J* = 2.0 Hz, H-8), 7.22 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.80 (2H, d, *J* = 8.4 Hz, H-3', 5'); ^{13}C NMR (acetone-d_6, 100 MHz) δ (ppm): 82.5 (d, C-2), 68.2 (d, C-3), 29.0 (t, C-4), 156.7 (s, C-5), 96.0 (d, C-6), 157.1 (s, C-7), 95.1 (d, C-8), 100.4 (s, C-9), 157.6 (s, C-10), 131.2 (s, C-1'), 129.5 (d, C-2', 6'), 158.0 (s, C-4'), 115.6 (d, C-3', 5'); ESI-MS: *m/z* 297 $[M+Na]^+$ (MF. $C_{15}H_{14}O_5$)。

(-)(2R,3R)Epiafzelechin (2)[6-7] Yellow crystals; mp 243 ~246℃, $[\alpha]_D^{20}$ −38.3° (*c* 0.23, acetone); ^{1}H NMR (acetone-d_6, 400 MHz) δ (ppm): 4.89 (1H, brs, H-2β), 4.17 (1H, m, H-3β), 2.69 (1H, dd, *J* = 3.6, 16.4 Hz, H-4α), 2.83 (1H, ddd, *J* = 4.4, 6.4 Hz, H-4β), 6.00 (1H, d, *J* = 2.4 Hz, H-6), 5.89 (1H, d, *J* = 2.4 Hz, H-8), 7.33 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.79(2H, d, *J* = 8.4 Hz, H-3', 5'); ^{13}C NMR (acetone-d_6, 100 MHz) δ (ppm): 79.3 (d, C-2), 66.7 (d, C-3), 28.9 (t, C-4), 157.0 (s, C-5), 96.0 (d, C-6), 157.4 (s, C-7), 95.4 (d, C-8), 99.5 (s, C-9), 157.5 (s, C-10), 131.2 (s, C-1'), 129.0 (d, C-2', 6'), 157.5 (s, C-4'), 115.1 (d, C-3', 5'); ESI-MS: *m/z* 297 $[M+Na]^+$ (MF. $C_{15}H_{14}O_5$)。

(-)(2R,3S)Afzelechin-(4α-8)(2R,3S)afzelechin (3)[8-9] Yellow amorphous powder; $[\alpha]_D^{20}$ −135.1° (*c* 0.09, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 4.55 (1H, d, *J* = 8.0 Hz, H-2β), 4.44 (1H, d, *J* = 7.2 Hz, H-2′β), 4.35 (1H, dd, *J* = 9.8, 4.8 Hz, H-3), 4.34 (1H, d, *J* = 6.1 Hz, H-4), 3.76 (1H, m, H-3′), 2.86 (1H, *J* = 16.2, 5.8 Hz, H-4′α), 2.51 (1H, *J* = 16.2, 8.7 Hz, H-4′β), 6.11(1H, s, H-6′), 5.92 (1H, d, *J* = 1.6 Hz, H-8), 5.84 (1H, d, *J* = 1.6 Hz, H-6), 6.98 (2H, d, *J* = 8.3 Hz, H-12, 16), 6.92 (2H, d, *J* = 8.3 Hz, H-12, 16'), 6.77 (2H, d, *J* = 8.5 Hz, H-13, 15), 6.67 (2H, d, *J* = 8.5 Hz, H-13′, 15′); ^{13}C

NMR (acetone-d_6, 125 MHz) δ (ppm): 82.6 (d, C-2), 73.8 (d, C-3), 38.6 (d, C-4), 157.3 (s, C-5), 96.33 (d, C-6), 158.0 (s, C-7), 96.9 (d, C-8), 107.2 (s, C-9), 155.7 (s, C-10), 132.2 (s, C-11), 129.6 (d, C-12, -16), 115.8 (d, C-13, -15), 158.1 (s, C-14), 83.7 (d, C-2′), 69.2 (d, C-3′), 29.6 (t, C-4′), 155.9 (s, C-5′), 97.4 (d, C-6′), 157.3 (s, C-7′), 108.2 (s, C-8′), 102.7 (s, C-9′), 155.1 (s, C-10′), 131.3 (s, C-11′), 130.1 (d, C-12′, -16′), 158.6 (s, C-14′), 115.8 (d, C-13′, -15′); ESI-MS *m/z*: 545 $[M-H]^-$(MF. $C_{30}H_{26}O_{10}$)。

(-)(2R,3S)Afzelechin-(4α-8)(2R,3R)epiafzelechin (4)[8-9] Yellow amorphous powder, $[\alpha]_D^{20}$ −206.0° (*c* 0.23, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm): 4.49 (1H, d, 7.3 Hz, H-2β), 4.63 (1H, m, H-2′β), 4.35 (1H, m, H-3), 4.37 (1H, d, *J* = 9.8 Hz, H-4), 4.09 (1H, m, H-3′), 2.95 (1H, *J* = 16.2, 4.8 Hz, H-4′α), 2.91 (1H, *J* = 16.2, 5.0 Hz, H-4′β), 6.15 (1H, s, H-6′), 5.99 (1H, d, *J* = 2.3 Hz, H-8), 5.94 (1H, d, *J* = 2.3 Hz, H-6), 7.07 (2H, d, *J* = 8.5 Hz, H-12, 16), 6.88 (2H, d, *J* = 8.5 Hz, H-12', 16'), 6.81 (2H, d, *J* = 8.5 Hz, H-13, 15), 6.62 (2H, d, *J* = 8.5 Hz, H-13′, 15′); ^{13}C NMR (acetone-d_6, 125 MHz) δ (ppm): 83.7 (d, C-2), 69.2 (d, C-3), 38.7 (d, C-4), 155.9 (s, C-5), 97.0 (d, C-6), 157.2 (s, C-7), 96.6 (d, C-8), 107.5 (s, C-9), 157.4 (s, C-10), 132.1 (s, C-11), 130.0 (d, C-12, -16), 115.8 (d, C-13, -15), 158.0 (s, C-14), 80.3 (d, C-2′), 67.8 (d, C-3′), 29.7 (t, C-4′), 155.7 (s, C-5′), 97.7 (d, C-6′), 156.3 (s, C-7′), 108.5 (s, C-8′), 101.7 (s, C-9′), 157.6 (s, C-10′), 131.3 (s, C-11′), 129.3 (d, C-12′, -16′), 158.5 (C-14′), 115.7 (d, C-13′, -15′); ESI-MS: *m/z* 568.8 $[M+Na]^+$ (MF. $C_{30}H_{26}O_{10}$)。

(+)catechin (5)[10] $C_{15}H_{14}O_6$, white powder, $[\alpha]_D^{20}$ 20.5° (*c* 0.08, acetone); ^{1}H NMR (acetone-d_6, 400 Hz) δ (ppm): 4.54 (1H, d, *J* = 7.6 Hz, H-2β), 3.97 (1H, m, H-3α), 2.51 (1H, dd, *J* = 8.4, 16.0 Hz, H-4β), 2.89 (1H, dd, *J* = 5.6, 15.6 Hz, H-4α), 6.01 (1H, d, *J* = 2.0 Hz, H-6), 5.86 (1H, d, *J* = 2.0 Hz, H-8), 6.88 (1H, d, *J* = 1.6 Hz, H-2'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.74 (1H, dd, *J* = 1.6, 8.0 Hz, H-6'); ^{13}C NMR (acetone-d_6, 100 MHz) δ (ppm): 82.7 (d, C-2), 68.3 (d, C-3), 28.8 (t, C-4), 157.2 (s, C-5), 96.1 (d, C-6), 156.9 (s, C-7), 96.1 (d, C-8), 100.6 (s, C-9), 157.7 (s, C-10), 132.2 (s, C-1'), 115.2 (d, C-2'), 145.7 (s, C-3'), 145.6 (s, C-4'), 115.7 (d, C-5'), 120.0 (d, C-6')。

Phaseic acid (6)[11] $C_{15}H_{20}O_5$, colorless crystals, mp 207 ~ 209℃, $[\alpha]_D^{20}$ −0.042° (*c* 0.16, MeOH); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 5.69 (1H, s, H-2), 7.98 (1H, d, *J* = 15.7 Hz, H-4), 6.44 (1H, d, *J* = 15.7 Hz, H-5), 2.02 (3H, s, H-6), 2.69 (1H, dd, *J* = 17.5, 2.1 Hz, H-3'a) , 2.79 (1H, d, *J* = 17.5 Hz, H-3′b), 2.28 (1H, dd, *J* = 19.5, 2.3 Hz, H-5′a), 2.31 (1H, dd, *J* = 19.5, 2.3 Hz, H-5'b), 1.07 (3H, s, H-7′), 3.52 (1H, d, *J* = 7.5 Hz, H-8′a), 3.78 (1H, dd, *J* = 7.5, 2.7 Hz, H-8′b), 0.87 (3H, s, H-9′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 167.0 (s, C-1), 118.7 (d, C-2), 149.0 (d, C-3), 130.6 (d, C-4), 133.4 (d, C-5), 20.8 (q, C-6), 81.2 (s, C-1′), 86.0 (s, C-2′), 53.0 (t, C-3′), 208.3 (s, C-4′), 51.9 (t, C-5′), 48.2 (s, C-6′), 19.1 (q, C-7′), 76.5 (t, C-8′), 15.3 (q, C-9′); HR-FABMS: *m/z* 280.1298 (cald. for $C_{15}H_{20}O_5$ 280.1305)。

Benzolic acid (**7**), 4-hydroxybenzoic acid (**8**), phloroglucinol (**9**), daucosterol (**10**), and β-sitosterol (**11**) were identified by comparing their data of EI-MS, ^{1}H NMR, ^{13}C NMR, and TLC with those of authentic compounds.

2.2 Anti-HSV activity

Flavans **1** ~ **4** were examined for their *in vitro* antiviral activity on HSV-1 and HSV-2 by MTT assay in cytopathic effect (CPE) tests[5]. Compounds **1**, **2**, ACV, and DMSO were non-cytotoxic below the concentrations of 0.65, 0.72, 0.100, and 6.30 mg mL^{-1}, respectively. The CC_{50} values of **1**, **2** and ACV were determined as 2.11, 1.75, and 0.99 mg mL^{-1}, respectively. Under non-cytotoxic concentrations, compounds **1** and **2**, when coexisted with the virus, moderately inhibited the CPE induced by HSV-1; however these four compounds were inactive to the replication of HSV-2 even at the maximum non-cytotoxic concentrations (2.37 and 2.63 mg mL^{-1} for **1** and **2**; both 1.46 mg mL^{-1} for **3** and **4**, respectively). The IC_{50} values of **1**, **2**, and ACV against HSV-1 were determined as 0.49, 0.55 mg mL^{-1}, and 2.64 μg mL^{-1},

respectively. The therapeutic indexes for compounds **1** and **2** against HSV-1 were 4.31 and 3.19, respectively. Flavans **1** and **2** could be recognized as bioactive constituents responsible for the anti-virus activity of *Folium Fici microcarpae* and recommended as reference markers for quality control of *Folium Fici microcarpae*[12].

References

[1] Jiang u New Medicine College. Encyclopedia of Chinese Materia Medica Vol. 2 [M]. Shanghai: Shanghai Science & Technology Press, 1977: 2528.(in Chinese)

[2] Chinese Pharmacopoeia Commission. Specifications Promulgated by the Ministry of Health, P R China Vol. 14 [M]. Beijing: People's Medical Publishing House, 1994: 182.(in Chinese)

[3] Chiang Y M, Su J K, Liu Y H, et al. New cyclopropyl-triterpenoids from the aerial roots of *Ficus microcarpa* [J]. Chem Pharm Bull, 2001, 49: 581−583.

[4] Lai Y Q, Zhou H B. Isolation and identification of the chemical constituents of *Folium Fici microcarpae* [J]. Food Drug, 2008, 10: 11−13.(in Chinese)

[5] Freshney R I. Animal Cell Culture, A Practical Approach [M]. London: Oxford University Press, 1992: 230−242.

[6] Shao Z Y, Zhu D Y, Guo Y W. A new flavan-3-ol glucoside from *Daphniphyllum oldhami* [J]. Chin Chem Lett, 2003, 14: 926−929.

[7] Min K R, Hwang B Y, Lim H S, et al. (-)-Epiafzelechin: Cyclooxygenase-1 inhibitor and anti-inflammatory agent from aerial parts of *Celastrus orbiculatus* [J]. Planta Med, 1999, 65: 460−462.

[8] Thompson R S, Jacques D, Haslam E, et al. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins [J]. J Chem Soc Perkin Trans 1, 1972(11): 1387−1399.

[9] Tanaka T, Nonaka G, Nishioka I. 7-O-galloyl-(+)-catechin and 3-O-galloylprocyanidin B-3 from *Sanguisorba officinalis* [J]. Phytochemistry, 1983, 22: 2575−2578.

[10] Lai Y F, Joseph J K. Procyanidin dimers and trimers from Douglas fir inner bark [J]. Phytochemistry, 1989, 28: 1743−1747.

[11] Hirai N, Kondo S, Ohigashi H. Deuterium-labelled phaseic acid and dihydrophaseic acid for internal standards [J]. Biosci Biotechn Biochem, 2003, 67: 2408−2415.

[12] Wu X P, Hu Y J, Lu Y Y, et al. Study on assay of the bioactive constituent (-)-epiafzelechin in the leaves of *Ficus microcarpa* L. [J]. Chin J Pharm Anal, 2008, 28(6): 992−995.(in Chinese)