

Prenylated stilbenes and flavonoids from the leaves of *Cajanus cajan*

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Available online 20 May, 2019

[ABSTRACT] Three new prenylated stilbenes, named as cajanusins A–C (**1–3**), and one new natural product cajanusin D (**4**), along with six known derivatives (**5–10**) were isolated from the leaves of *Cajanus cajan*. Their structures were fully elucidated by means of extensive spectroscopic methods and comparison with data in the reported literatures. The new compounds of **1** and **2** were evaluated for *in vitro* cytotoxic activities against a panel of human cancer cell lines.

[KEY WORDS] *Cajanus cajan*; Stilbenes; Flavonoids; Leguminosae

[CLC Number] R284.1 **[Document code]** A **[Article ID]** 2095-6975(2019)05-0381-06

Introduction

The plant *Cajanus cajan* is one of the representative genera in the Leguminosae family. It is an India native green shrub growing for many years and well-known as pigeon bean, pigeon pea, soybean, willow bean, clover bean, and millennium bean. This plant derive from India, and now, it has been commercially cultivated throughout tropical and subtropical regions of the world^[1-2], as well as widely used as medicinal

plant, food, fodder, and environmental protection plant^[3]. Plants in the genus *Cajanus* are known for their enriched flavonoid and stilbene contents^[4-7], which are responsible for a variety of the biological activities for these herbal medicines^[4-5]. Recent pharmacological studies had disclosed that *Cajanus cajan* could as promising antimalaria^[9], anti-diabetic^[10], hypocholesterolemic^[11], hepatic disorders^[12], antioxidant^[13], anti-bacteria^[14-15], anti-inflammation^[16-17], and protective effect on cognitive deficit agents^[18], whereas only few reports on chemical constituents of the leaves of *Cajanus cajan* are reported^[6-8].

Motivated by an ongoing research program focusing on biologically meaningful and structurally diverse natural products from the medicinal plants, we recently have reported a series of acylphloroglucinols with antibacterial or antitumor activities^[19-22]. The phytochemical study was carried out on the plant *Cajanus cajan* and resulted in the isolation of four new stilbenes, designed as cajanusins A–C (**1–3**) and cajanusin D (**4**), along with six known derivatives 5-hydroxy-6, 7-(2", 2"-dimethylchromene)flavanone (**5**)^[23], longistyline A (**6**)^[24-25], longistyline C (**7**)^[26], cajanolactone A (**8**)^[27], pinnostrobin (**9**)^[27], and cajanonic acid A (**10**)^[5] (Fig. 1). Herein, the isolation, structural elucidation of compounds **1–4**, and anti-tumor activities of **1–2** are described.

[Received on] 18-Dec.-2018

[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81773602 and 81502949), the Natural Science Foundation of Guangdong Province (Nos. 2016A030313149 and 2016A020217015), the Innovative Team Project of GZUCM (No. 2016KYTD04), Pearl River Science and Technology New Star Fund of Guangzhou (No. 201605120849569), Guangdong Special Support Program (No. 2017TQ04R599), and the Foundation of Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences.

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These authors have no conflict of interest to declare.

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Results and Discussion

Compound **1** was obtained as a white pinnate. Its molecular formula was assigned as $C_{21}H_{24}O_3$ referring to the high-resolution electrospray ionization mass spectroscopy (HR-ESI-MS) in negative mode, wherein an ion peak was observed at m/z 323.1652 $[M - H]^-$ (Calcd. for $C_{21}H_{23}O_3$, 323.1647). The IR spectrum showed a series of characteristic absorptions for the hydroxyl (3356 cm^{-1}) and carbonyl (1634 cm^{-1}) functional groups. The ^1H and ^{13}C NMR spectra of **1** displayed the signals responsive for an active free hydroxyl (δ_{H} 12.67, 1H, s), an aldehyde [δ_{H} 10.07 (1H, s), δ_{C} 193.2], a mono-substituted benzene, a methoxyl [δ_{H} 3.88, (3H, s), δ_{C} 55.8], and two methyl [δ_{H} 1.69 and 1.76 (each 3H, s), δ_{C} 25.7 and 18.0] functionalities.

The ^1H - ^1H COSY spectrum of **1** revealed the presence of

three spin-coupling fragments as shown in Fig. 2, namely, **a** H-1'' to H-2'', **b** H-7 to H-8, and **c** H-2'/H-3'/H-4'/H-5'/H-6'. The critical HMBC correlations from H-3' to C-2'/C-1' and H-4' to C-3', indicated that **1** possessed a monosubstituted benzene ring B (Fig. 2). Besides, the HMBC cross-peaks between H-2' and C-4'/C-8, H-8 and C-7/C-2'/C-1'/C-1, as well as H-7 and C-8/C-6/C-2'/C-1'/C-1' collectively pointed to that the ring systems A and B were connected through C-1 and C-1' via the remaining unassigned two mutually-coupled CH_2 units, formatting a typical stilbene skeleton. The presence of isopentene moiety could be verified by the unambiguous HMBC correlations from H-1'' to C-2''/C-3''/C-1'/C-5, H-4'' to C-5''/C-3''/C-2'', coupling with H-5'' to C-4''/C-3''/C-2'', and it could be further evidenced to attach at the C-6 position in the ring A attributed to the conclusive correlations from H-2'' and H-1'' to C-6 (Fig. 2).

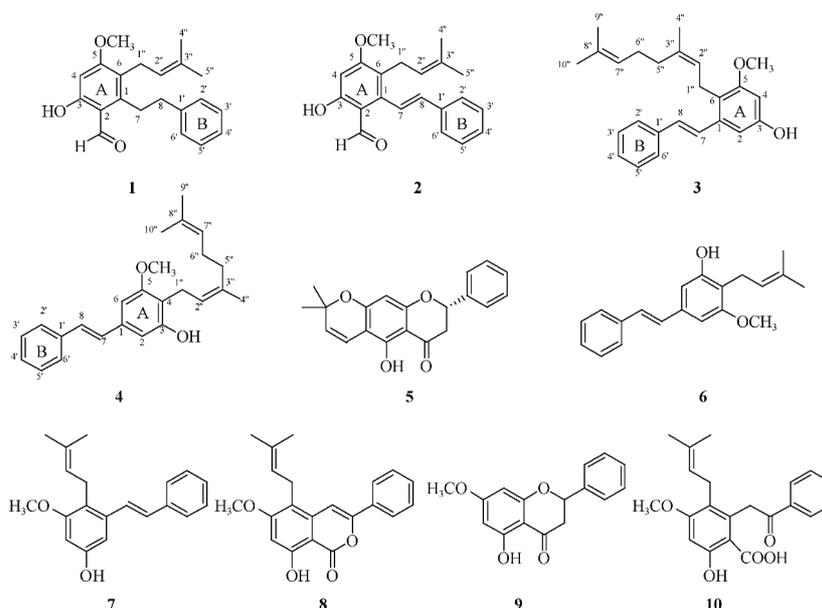


Fig. 1 Structures of compounds 1–10

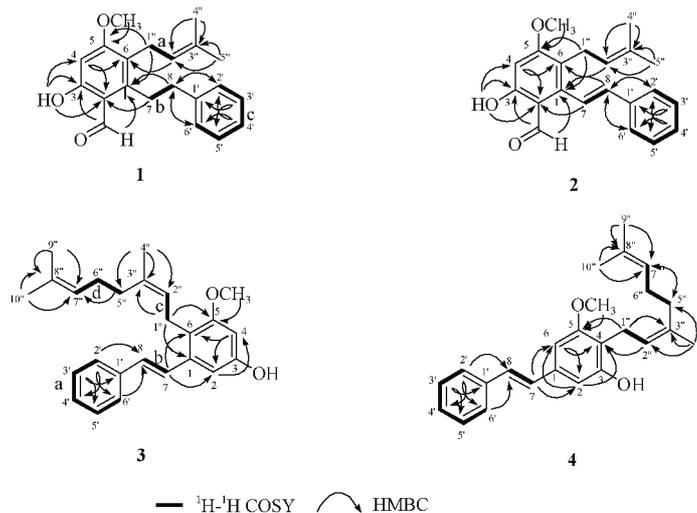


Fig. 2 Key ^1H - ^1H COSY and HMBC correlations of 1–4

With a careful inspection of the residual HMBC correlations, the obvious correlations of the proton for the aldehyde functional group (δ_{H} 10.07) to C-4/C-3/C-2 were successfully distinguished, which provided the direct evidence to locate the aldehyde group at C-2 position (Fig. 2). Moreover, the free hydroxyl (δ_{H} 12.67) and the methoxyl (δ_{H} 3.88) functional groups could be readily ascertained to the C-3 and C-5 positions in the ring A respectively, because of the informative HMBC correlations from 3-OH to C-4/C-3/C-2 and 5-MeO (δ_{H} 3.88) to C-5 (Fig. 2). Therefore, the structure of compound **1** was successfully established to be cajanusin A as a novel stilbene derivative with a rarely nature-occurring aldehyde functionality.

Compound **2** was separated as a faint yellow oil. The molecular formula of **2** was determined to be $\text{C}_{21}\text{H}_{22}\text{O}_3$ based on a quasi-molecular ion peak at m/z 321.1480 $[\text{M} - \text{H}]^-$ (Calcd. for $\text{C}_{21}\text{H}_{21}\text{O}_3$, 321.1480) in its HR-ESI-MS spectrum, suggesting two indices of hydrogen deficiency less than those of compound **1**. The IR spectrum showed characteristic absorptions corresponding to a hydroxyl (3356 cm^{-1}) and a carbonyl (1717 cm^{-1}) moieties. A closer comparison of its ^1H and ^{13}C NMR spectra (Table 1) with those of **1** suggested that compound **2** should also share a classic stilbene scaffold due to the similarity of their NMR profiles in most cases (Table 1).

Table 1 ^1H (500 MHz) and ^{13}C NMR (125 MHz) data of **1** and **2** in CDCl_3

| No. | 1 | | 2 | |
|-----|-------------------------------|---------------------|-------------------------------|---------------------|
| | δ_{H} (J in Hz) | δ_{C} | δ_{H} (J in Hz) | δ_{C} |
| 1 | | 144.3, C | | 142.9, C |
| 2 | | 112.1, C | | 113.2, C |
| 3 | | 165.2, C | | 164.3, C |
| 4 | 6.34, s | 97.4, CH | 6.41, s | 97.9, CH |
| 5 | | 165.2, C | | 164.6, C |
| 6 | | 121.2, C | | 121, 1, C |
| 7 | 3.15, dd, 6.9, 9.7 | 29.5, CH_2 | 7.23, d, 16.4 | 122.9, CH |
| 8 | 2.87, dd, 6.9, 9.7 | 38.5, CH_2 | 6.56, d, 16.4 | 138.5, CH |
| 1' | | 140.7, C | | 136.3, C |
| 2' | 7.17, d, 7.8 | 128.3, CH | 7.50, d, 7.6 | 126.6, CH |
| 3' | 7.32, t, 7.8 | 128.6, CH | 7.40, t, 7.6 | 128.8, CH |
| 4' | 7.24, t, 7.8 | 126.5, CH | 7.33, t, 7.6 | 128.5, CH |
| 5' | 7.32, t, 7.8 | 128.6, CH | 7.40, t, 7.6 | 128.8, CH |
| 6' | 7.17, d, 7.8 | 128.3, CH | 7.50, d, 7.6 | 126.6, CH |
| 1'' | 3.31, d, 6.4 | 24.1, CH_2 | 3.33, d, 6.8 | 25.8, CH_2 |
| 2'' | 5.01, m | 122.9, CH | 5.07, m | 122.2, CH |
| 3'' | | 131.6, C | | 131.6, C |
| 4'' | 1.76, s | 18.0, CH_3 | 1.69, s | 17.9, CH_3 |
| 5'' | 1.69, s | 25.7, CH_3 | 1.68, s | 25.2, CH_3 |
| CHO | 10.07, s | 193.2, CH | 10.00, s | 195.3, CH |
| MeO | 3.88, s | 55.8, CH_3 | 3.90, s | 55.9, CH_3 |

The most notable difference between them was that the two methylenes at C-7 (δ_{C} 29.5) and C-8 (δ_{C} 38.5) in **1** was replaced by a trans double bond at C-7 (δ_{C} 122.9) and C-8 (δ_{C} 138.5) in **2**, which could be further reinforced by the informative HMBC cross-peaks of H-7 (δ_{H} 7.23) and H-8 (δ_{H} 6.56) to C-1 (δ_{C} 142.9) and C-1' (δ_{C} 136.3). Therefore, the structure of compound **2** was finally assigned to be a new stilbene aldehyde derivative and given the trivial name as cajanusin B.

Compound **3** was also isolated as faint yellow oil, and its molecular formula was deduced to be $\text{C}_{25}\text{H}_{30}\text{O}_2$ on the basis of the positive HR-ESI-MS mode with a pseudo-molecular peak found at m/z 363.2315 $[\text{M} + \text{H}]^+$ (Calcd. for $\text{C}_{25}\text{H}_{31}\text{O}_2$, 363.2315). The special resonances of the ^1H and ^{13}C NMR spectra of **3** also collectively pointed to a stilbene fragment with various moieties including a hydroxyl, a monosubstituted benzene ring, a methoxyl [δ_{H} (3.81, 3H, s), δ_{C} (55.7)], and three methyl [δ_{H} (1.80, 3H, s), (1.62, 3H, s), and (1.55, 3H, s); δ_{C} 16.2, 25.6, and 17.6] functionalities. Based on these informative results, compound **3** was also rationally assumed to be a stilbene analogue.

The construction of the gross structure for compound **3** was accomplished by the extensive interpretation of 2D NMR spectra, in particular with HMBC spectrum. Firstly, the ^1H - ^1H COSY spectrum of **3** suggested the presence of four spin systems: **a** (H-2'/H-3'/H-4'/H-5'/H-6'), **b** (H7/H-8), **c** (H-1''/H-2''), and **d** (H-5''/H-6''/H-7'') as drawn with black bold lines in Fig. 2. On the basis of fragment **b**, the conclusive HMBC correlations from H-2' (H-6') to C-4', and H-3' (H-5') to C-1' clarified that **3** should possess the monosubstituted benzene ring B (Fig. 2). Besides, the HMBC correlations between H-2 and C-4 as well as H-4 and C-2/C-6 suggested that the ring A was a 1,3,5,6-tetrasubstituted benzene ring with one free hydroxyl and one methoxyl functionalities at C-3 and C-5 positions respectively, based on the informative HMBC correlations from methoxyl group 5-MeO to C-5 and the sharply low-field shift for C-3 (δ_{C} 154.4) and C-5 (δ_{C} 158.6). Furthermore, the critical HMBC correlations from H-7 to C-8/C-1/C-1' as well as H-8 to C-7/C-1/C-1', along with the fragment **b**, suggested that the two aromatic rings A and B were connected through C-1 and C-1' via a double bond. The aforementioned evidence further confirmed that this compound ought to be belonged to the stilbene family.

In addition, the HMBC correlation from 5-MeO (δ_{H} 3.81) to C-5 verified the location of the methoxyl group at C-5 position (Fig. 2). Moreover, on the basis of fragments **c** (C-1''/C-2'') and **d** (C-5''/C-6''/C-7''), the key HMBC correlations from H-9'' to C-7'' and C-8'', H-10'' to C-7'' and C-8'', H-4'' to C-2''/C-3''/C-5'', H-5'' to C-2'' and C-7'' (Fig. 2) certified that there were two isopentene nucleuses existing in the molecule and they were linked together via C-5'' position, giving rise to a farnesyl fragment. The farnesyl unit could be further unambiguously rationalized to be substituted at C-6 position of ring A based on the HMBC correlations from H-1'' to C-1 and C-5. Therefore, the structure of **3** was fully

characterized and established as shown in Fig. 2 with the name as cajanusin C.

Compound **4** was also obtained as yellow oil. It has the same molecular formula with eleven degrees of hydrogen deficiency as **4** based on the positive HR-ESI-MS at m/z 363.2319 $[M + H]^+$ (Calcd. for $C_{25}H_{31}O_2$, 363.2319). The IR spectrum showed characteristic absorptions for benzene ring (1451 cm^{-1}) and monosubstituted benzene ring (692 cm^{-1}), respectively. After a careful analysis of its 1D and 2D NMR spectra, it could be readily discovered that the NMR spectroscopic data, HMBC (Table 2), and COSY correlations observed for compound **4** exhibited almost the same results as those of **3**, except for that the placement of the farnesyl substituent at C-6 position in **3** was transformed to the C-4 position in **4** in the tetra-substituted benzene unit. The aforementioned deduction could be further supported by the key HMBC correlations from H-1" to C-3 (δ_C 155.7) and C-5 (δ_C 158.0). Therefore, the structure of compound **4** was completely interpreted, and suggested to be a regioisomer of the stilbene derivative **3**, which was previously synthesized by Jiang's group [29], and designed as a new natural product in our study with the name as cajanusin D.

Table 2 ^1H (500 MHz) and ^{13}C NMR (125 MHz) data of **3** and **4** in CDCl_3

| No. | 3 | | 4 | |
|-----|----------------------|---------------------|----------------------|---------------------|
| | δ_H (J in Hz) | δ_C | δ_H (J in Hz) | δ_C |
| 1 | | 137.6, C | | 137.3, C |
| 2 | 6.70, d, 2.1 | 104.1, CH | 6.68, d, 1.5 | 107.3, CH |
| 3 | | 154.4, C | | 155.7, C |
| 4 | 6.39, d, 2.5 | 98.5, CH | | 115.0, C |
| 5 | | 158.6, C | | 158.0, C |
| 6 | | 121.1, C | 6.65, d, 1.5 | 101.6, CH |
| 7 | 6.96, d, 5.9 | 126.6, CH | 7.04, overlapped | 128.7, CH |
| 8 | 6.92, d, 5.9 | 126.6, CH | 7.04, overlapped | 128.7, CH |
| 1' | | 138.1, C | | 136.7, C |
| 2' | 7.48, d, 7.4 | 128.7, CH | 7.51, d, 7.7 | 128.4, CH |
| 3' | 7.36, t, 7.4 | 126.6, CH | 7.36, t, 7.7 | 127.5, CH |
| 4' | 7.26, t, 7.4 | 127.6, CH | 7.25, t, 7.7 | 126.5, CH |
| 5' | 7.36, t, 7.4 | 126.6, CH | 7.36, t, 7.7 | 127.5, CH |
| 6' | 7.48, d, 7.4 | 128.7, CH | 7.51, d, 7.7 | 128.4, CH |
| 1" | 3.44, d, 6.4 | 24.3, CH_2 | 3.44, d, 7.1 | 22.3, CH_2 |
| 2" | 5.12, m | 123.6, CH | 5.26, m | 121.7, CH |
| 3" | | 134.5, C | | 138.4, C |
| 4" | 1.80, s | 16.2, CH_3 | 1.82, s | 16.2, CH_3 |
| 5" | 1.98, m | 39.7, CH_2 | 2.11, m | 39.7, CH_2 |
| 6" | 2.06, m | 26.7, CH_2 | 2.05, m | 25.7, CH_2 |
| 7" | 5.06, m | 124.3, CH | 5.08, m | 123.9, CH |
| 8" | | 131.3, C | | 131.9, C |
| 9" | 1.55, s | 17.6, CH_3 | 1.60, s | 17.7, CH_3 |
| 10" | 1.62, s | 25.6, CH_3 | 1.69, s | 26.5, CH_3 |
| MeO | 3.81, s | 55.7, CH_3 | 3.88, s | 55.8, CH_3 |

Compounds **1** and **2** were preliminary evaluated for their cytotoxicities against a panel of human cancer cell lines MCF-7, HeLa, OVCAR-3, C33A, T47D, and HepG-2 with paclitaxel as the positive control. The new compounds **1** and **2** showed weak cell growth inhibitory activities in MCF-7 cells with IC_{50} values as 86.6 ± 7.5 and $77.6 \pm 0.3\ \mu\text{mol}\cdot\text{L}^{-1}$, respectively. For these IC_{50} , they are non-cytotoxic.

Experimental

General experimental procedures

UV spectra were measured on a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). IR data were measured on a Shimadzu IR Affinity-1 spectrometer (Shimadzu, Kyoto, Japan). 1D and 2D NMR spectra were recorded on a Bruker Avance-500 spectrometer (Bruker, Fällanden, Switzerland) with TMS as internal standard, δ in ppm, J in Hz. HR-EI-MS was measured on a Thermo MAT95XP high resolution mass spectrometer and EI-MS on a Thermo DSQ EI mass spectrometer (Thermo Scientific, Massachusetts, USA). A Hitachi Primaide (Hitachi Instruments (Dalian) Co., Ltd.) equipped with a diode array detector (DAD) using a preparative YMC ODS C_{18} column (20 mm \times 250 mm, 5 μm) was used for preparative HPLC separation. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China) was used for column chromatography, Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden), and reversed-phase C_{18} (150–200 mesh, Merck, Germany) were used to perform column chromatography (CC). TLC analysis was carried out on silica gel plates (Merck KGaA, Darmstadt, Germany). All reagents were provided by Tianjin Damao Chemical Co. (Tianjin, China).

Human cervical cancer cell line HeLa and breast cancer cell line MCF-7 were obtained from professor Huang Ying, American State University. Ovarian cancer cell line OVCAR 3, human cervical carcinoma cell line C33A, breast cancer cell line T47D, liver cancer cell line HepG2 were gained from Cell Resource Center, Shanghai Institute of Life Sciences, Chinese Academy of Sciences.

Plant material

The leaves of *Cajanus cajan* were collected from Wenshan County, Yunnan Province, China in July 2016 and authenticated by Dr. DENG Yun-Fei of South China Botanical Garden. A voucher specimen (No# SCBG-NP-1997-18) was deposited at the Laboratory of Natural Product Medicinal Chemistry, South China Botanical Garden.

Extraction and isolation

Air-dried leaves of *Cajanus cajan* (25 kg) were extracted three times with 95% EtOH. The concentrated ethanolic extract was scattered with 50–60 °C hot water (50 L) and placed at room temperature (26 °C) for 8 h. After centrifugation, the precipitate was mixed with water (5 L) and extracted with dichloromethane to yield dichloromethane soluble extract (435 g). The dichloromethane-soluble extract (435 g) was subjected to silica gel column chromatography eluted with a

gradient mixture of *n*-hexane–EtOAc (50 : 1 to 1 : 1, *V/V*) to afford give 190 fractions, which were combined into 10 fractions (J.1–J.10) after TLC analysis. J.4 was subjected to ODS column chromatography using (MeOH–H₂O) (90 : 10 to 100 : 0, *V/V*) as eluent to yield 5 fractions (J.4.1–J.4.5). J.4.1 was subjected to a silica gel column chromatography (*n*-hexane–acetone, 20 : 1 to 10 : 1, *V/V*) to give another 4 fractions (J.4.1.1–J.4.1.4), and J.4.1.2 was further purified by reversed phase semi-preparative HPLC (MeOH–H₂O, 83 : 17, 3 mL·min⁻¹) to afford compounds **6** (5.12 mg), **9** (12.39 mg), **3** (2.60 mg), and **4** (3.90 mg). J.4.2 was separated by a Sephadex LH-20 CC using MeOH as eluent and semi-preparative HPLC using MeOH–H₂O (90 : 10, *V/V*, 3 mL·min⁻¹) as eluent to afford compounds **5** (3.89 mg), **1** (3.89 mg), **2** (4.84 mg), **7** (1.10 mg), and **8** (5.28 mg). The chemical isolation of fraction J.9 was conducted by ODS column with (MeOH–H₂O, 50 : 50 to 100 : 0, *V/V*) as eluent to yield a yellow powder, the powder was repeatedly washed with ethanol and filtered for several times to obtain the white powder **10** (880.00 mg).

cajanusin A (**1**)

White pinnate; UV (MeOH) λ_{\max}/nm (log ϵ) 235 (3.20), 287 (2.88); IR ν_{\max} : 3356, 2959, 2920, 2851, 1634, 1456, 1371, 1325, 1287, 1223, 1204, 1134, 814, 799, 747, 698 cm⁻¹. ¹H NMR (500 MHz) and ¹³C NMR (125 MHz) data, see Table 1; negative ESI-MS m/z 323 [M – H]⁻; HR-ESI-MS m/z 323.1652 [M – H]⁻ (for C₂₁H₂₃O₃, Calcd. 323.1647).

cajanusin B (**2**)

Yellow oil; UV (MeOH) λ_{\max}/nm (log ϵ) 268 (3.99), 339 (3.38); IR ν_{\max} : 3356, 2955, 2924, 2851, 1732, 1717, 1614, 1456, 1364, 1281, 1132, 1072, 1016, 741, 692, 598, 554 cm⁻¹. ¹H (500 MHz) and ¹³C NMR (125 MHz) data, see Table 1; negative ESI-MS m/z 321 [M – H]⁻; HR-ESI-MS m/z 321.1480 [M – H]⁻ (for C₂₁H₂₁O₃, Calcd. 321.1480).

cajanusin C (**3**)

Yellow oil; UV (MeOH) λ_{\max}/nm (log ϵ) 202 (3.46), 295 (2.90) nm; IR ν_{\max} : 3335, 3263, 2924, 2845, 1599, 1584, 1456, 1435, 1375, 1314, 1225, 1190, 1144, 1072, 1055, 1017, 962, 831, 745, 731, 692 cm⁻¹. ¹H (500 MHz) and ¹³C NMR (125 MHz) data, see Table 1; positive ESI-MS m/z 363 [M + H]⁺; HR-ESI-MS m/z 363.2315 [M + H]⁺ (for C₂₅H₃₁O₂, Calcd. 363.2315).

cajanusin D (**4**)

Yellow oil; UV (MeOH) λ_{\max}/nm (log ϵ) 315 (4.03); IR ν_{\max} : 2967, 2924, 1609, 1582, 1508, 1451, 1420, 1375, 1319, 1296, 1260, 1223, 1165, 1094, 1028, 959, 862, 816, 750, 692, 625 cm⁻¹. ¹H (500 MHz) and ¹³C NMR (125 MHz) data, see Table 1; positive ESI-MS m/z 363 [M + H]⁺; HR-ESI-MS m/z 363.2319 [M + H]⁺ (for C₂₅H₃₁O₂, Calcd. 363.2319).

Cytotoxicity assay

Human cervical cancer cell line HeLa, breast cancer cell line MCF-7, ovarian cancer cell line OVCAR 3 were grown in RPMI 1640 containing 10% FBS, while human cervical carcinoma cell line C33A, breast cancer cell line T47D, liver

cancer cell line HepG2 were grown in DMEM containing 10% FBS at 37 °C, 5% CO₂, saturated humidity. To evaluate the cytotoxicity of compounds **1** and **2**, the cells (100 μL) with a density of 5×10^4 cells/mL of media were seeded into a 96-well plates and incubated for 24 h, and then treated with different concentrations (100, 50, 25, 12.5, 6.25, and 3.125 $\mu\text{mol}\cdot\text{L}^{-1}$) of the test compounds for 48 h, at the cell culture conditions. After that, the cell monolayers were fixed by 15% (*W/V*) trichloroacetic acid (4 °C, 1 h) and stained for 10 min by 0.4% (*W/V*) SRB, which was dissolved in 1% acetic acid. The unbound dye was removed by washing repeatedly with 1% acetic acid, and then dissolved into the protein-bound dye in 10 mmol·L⁻¹ Tris base solution (100 μL) for OD determination at 515 nm using a microplate reader. Paclitaxel was used as the positive control, while cells without any treatment were set as drug negative control. After that, cell survival in each well was monitored by Sulforhodamine B (SRB) assay [28]. All data were obtained in triplicate and presented as means \pm SD. Moreover, IC₅₀ values (concentration to inhibit 50% of cell growth) were calculated by the SigmaPlot 10.0 software with the use of a non-linear curve-fitting method.

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Cite this article as: WU Gui-Yun, ZHANG Xiao, GUO Xue-Ying, HUO Lu-Qiong, LIU Hong-Xin, SHEN Xiao-Ling, QIU Sheng-Xiang, HU Ying-Jie, TAN Hai-Bo. Prenylated stilbenes and flavonoids from the leaves of *Cajanus cajan* [J]. *Chin J Nat Med*, 2019, **17**(5): 381-386.