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Preparative isolation of four new and two known flavonoids from the leaf of *Patrinia villosa* Juss. by counter-current chromatography and evaluation of their anticancer activities in vitro

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Abstract

A preparative counter-current chromatography (CCC) was used to isolate and separate chemical constituents from the leaf of *Patrinia villosa*, a famous traditional Chinese medicinal herb. Six flavonoids including two known and four novel compounds were successfully simultaneous purified by CCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (10: 13: 13: 10, v/v) by increasing the flow rate of the mobile phase from 1.0 ml/min to 2.0 ml/min after 110 min to bring out the late eluters. The separation produced total of 44.9 mg fraction I with 99.1% purity, 35.5 mg fraction II with 98.8% purity, 79.8 mg fraction III with 99.3% purity, 45.8 mg fraction IV with 98.8% purity, 39.8 mg fraction V with 98.6% purity and 9.6 mg fraction VI with 97.5% purity from 400 mg crude extract in one single isolation procedure and less than 10 h, and the obtained fractions were all analyzed by high performance liquid chromatography (HPLC). Their chemical structures were elucidated as (2*S*)-5,7,2′,6′-tetrahydroxy-6,8-di (γ,γ-dimethylallyl) flavanone (**1**), (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone (**2**), (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone (**3**), (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethylpyrano [5″, 6″: 6, 7] flavanone (**4**), (2*S*, 3″*S*)-5,2′,6′-trihydroxy-3″-γ,γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydropyrano [5″, 6″: 6, 7] flavanone (**5**) and licoagrochalcone B (**6**), respectively, by spectrum methods including UV, IR, high resolution (HR)-electrospray ionization (ESI)-MS, 1-dimension (1D) and 2-dimension (2D) NMR techniques. Among them, compounds **2**, **3**, **4**, and **5** were new compounds and discovered from nature for the first time. The recoveries of the six compounds were 91.2%, 91.4%, 92.1%, 90.5%, 90.3% and 89.7%, respectively, in CCC step. Subsequently, their anticancer activities were also examined to inhibit human cancer cells' growth including A549, BEL-7402, SGC-7901, MCF-7, HT-29, K562 and A498 cell lines by MTT method in vitro. The results indicated that the compounds **1**, **2** and **3** exhibited high anticancer activities ($IC_{50} < 7$ μg/ml), especially to K562 cancer cell ($IC_{50} < 3.1$ μg/ml), and the compounds **4**, **5** and **6** exhibited weaker inhibition effect ($IC_{50} < 30$ μg/ml).

Keywords: *Patrinia villosa* Juss.; Preparative chromatography; Counter-current chromatography; Flavonoid

1. Introduction

Patrinia, a genus of about 20 species, is mainly distributed in central to east of Asia and northeast of North America, 10 of which grows in China. *Patrinia* species have been used as medicinal plants for more than 2000 years from *ShenNongBenCaoJing*, a famous ancient Chinese medicinal literary, and some of them still are used in folk medicine as anti-virus and anti-bacteria [1,2], especially two species, *P. scabiosaefolia* Fisch and *Patrinia villosa* (*BaiJiangCao* in Chinese).

With regard to the chemical constituents of this genus, except for some iridoids [3,4], we have isolated and separated two C-glycosylflavones (isovitexin and isoorientin) [5], a peptide derivative aurentiamide acetate [6] and several prenylated flavonoids [7,8] from it. A literature search did not yield any more references to early report on study of chemicals from the leaf of the medicinal herb *P. villosa*. So, further chemical research and discovery from the leaf of *P. villosa* is warranted for exploiting new traditional Chinese medicine (TCM) products and pharmacological tests.

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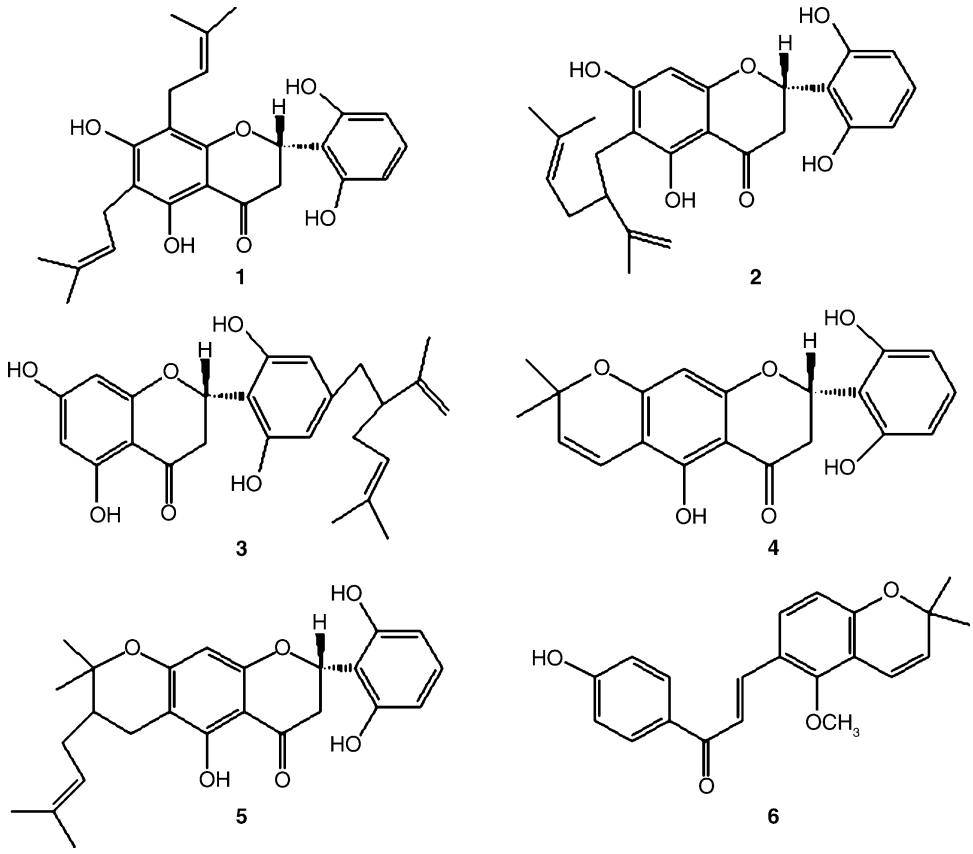

Fig. 1. The chemical structures of the six compounds.

Generally, some conventional methods including silica gel, polyamide and preparative RPLC are often used to isolate pure products from medicinal plants, but they are tedious, time consuming, requiring multiple chromatographic steps. Counter-current chromatography (CCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support, has an excellent sample recovery. So, it has been successfully applied to isolate and purify chemicals from natural materials [9–12].

In the present paper, a preparative CCC was used as a tool to isolate and separate chemicals from the leaf of *P. villosa* and six compounds were simultaneously separated in only one CCC run. Their chemical structures were elucidated as (2*S*)-5,7,2′,6′-tetrahydroxy-6,8-di (γ,γ-dimethylallyl) flavanone (**1**), (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone (**2**) (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone (**3**), (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethylpyrano [5″, 6″: 6, 7] flavanone (**4**), (2*S*,3″*S*)-5,2′,6′-trihydroxy-3″-γ,γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydropyrano [5″, 6″: 6, 7] flavanone (**5**) and licoagrochalcone B (**6**), respectively (shown in Fig. 1). Among them, compounds **2**, **3**, **4**, and **5** were new compounds and discovered from nature for the first time. Subsequently, the anticancer activities of the obtained compounds to inhibit human cancer cell lines' growth were evaluated by 3,-4,5-dimethyliazol-2,5-diphenyl tetrazolium bromide (MTT) method in vitro.

2. Experimental

2.1. Apparatus

Preparative CCC was carried out with a model TBE–300A counter-current chromatograph (Shenzhen, Tauto Biotech, China). The apparatus was equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (*R*) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The CCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 280 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

2.2. Reagents

n-Hexane, ethyl acetate, methanol, ethanol and acetic acid were analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. While acetonitrile used for HPLC was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q

water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The leaf of *P. villosa* was purchased from a local drug store and identified by Doctor Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

2.3. Preparation of the crude extract for CCC isolation

The leaf of *P. villosa* was ground into powder, 3.0 kg of the powder was extracted by reflux with 3.0×10^4 ml 75% aqueous ethanol for two times. The mixture was filtered, and 2.1×10^4 ml filtrate was collected. The extract was then concentrated to no ethanol by rotary vaporization at 60 °C under reduced pressure and 600 ml residue was obtained. Then the residue was redissolved in water (total volume 1500 ml), which was added into a glass column (6.0 cm × 60 cm, contained 2.0 kg AB-8 macroporous resin, Nankai University, Tianjin, China). Five thousand millilitres water was first used to elute the resin until the elution was nearly no color, and 3000 ml 30% aqueous ethanol was used to elute the resin, too. Then 6000 ml 85% aqueous ethanol was used to elute the target compounds, and 20 elution fractions (300 ml for each) were collected and 6 (from 5 to 10 fractions) were united and evaporated to dryness according to HPLC analysis, which was used for CCC isolation and separation.

2.4. Preparation of two-phase solvent system and sample solution

In the present paper, we selected several kinds of two-phase solvent systems. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. The sample solution was prepared by dissolving the crude extract in the solvent mixture of lower phase and upper phase (1: 1, v/v) of the solvent system for isolation because the sample was not easily dissolved in either phase.

2.5. CCC separation procedure

In each separation, the coil column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 900 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 1.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, approximately 10 ml of the sample solution containing 400 mg of the crude extract was injected into the head of the column through the injection valve. After 110 min, the flow rate of the mobile phase was increased to 2.0 ml/min. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm and the separation temperature was controlled at 35 °C. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis and identification of CCC fractions

The analytical HPLC system used throughout this study consisted of 515 pump and 2487 detector (Waters), and a model N2000 workstation (Zhejiang University, Hangzhou, China). The crude sample and peak fractions obtained by HSCCC were analyzed by HPLC. The column used was a reversed-phase Lichrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm) (Hanbang Science, Jiang Su Province, China) with a pre-column equipped with the same stationary phase, the mobile phase was CH_3CN–MeOH–H_2O–HAC (40: 25: 35: 2, v/v/v). The flow rate was 0.8 ml/min, and the effluent was monitored at 280 nm and the column temperature was set at 30 °C.

Identification of CCC fractions was carried out by Shimadzu UV 210A spectrometer (Japan), IR spectra (Hitachi 275-50), MS (Finnigan MAT 711), 1-dimension (1D) and 2-dimension (2D) NMR spectra (Varian Unity Inova–500).

2.7. Anticancer assay

MTT assay was performed as described [13]. Briefly, cells were seeded at a concentration of 1.5×10^5 cells/ml in a 96 well plates. After overnight incubation, serial concentrations of the compounds were added. Serial concentrations of test sample were prepared by dissolving the compound in dimethyl sulfoxide (DMSO) followed by dilution with RPMI-1640 medium to yield the final DMSO concentration in the assay well as 0.2%. Each concentration was repeated three times. These cells were incubated in a humidified atmosphere with 5% CO_2 for 3 days. Then 20 μl MTT solution (4.2 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was removed and formazan was dissolved in DMSO and the optical density was measured at 590 nm using a Bio-assay reader (Bio Rad, USA). The growth inhibition was determined using: Growth inhibition = (Control O.D. − Sample O.D.)/Control O.D., and IC_{50}, which is the drug concentration resulting in a 50% inhibition of cell growth, is calculated from dose–inhibition curves.

3. Results and discussion

3.1. HPLC conditions

First, the crude extract used for further CCC isolation was analyzed by HPLC. So, a good HPLC condition was required. In our research, different mobile phases (methanol–water, acetonitrile–water, methanol–acetonitrile–water) with different concentration of acetic acid, different flow rates, detection wavelength and column temperature were all tested. The result indicated that the mobile phase was composed of CH_3CN–MeOH–H_2O–HAC at the volume ratio of 40: 25: 35: 2 (v/v), and the flow rate, column temperature and detection wavelength were set at 0.8 ml/min, 30 °C and 280 nm, which were most suitable for our analysis. Under the above conditions, a satisfactory separation of the target compounds was obtained, and the HPLC chromatogram of the crude extract is given in Fig. 2, which mainly contained six peaks, and peaks **1**, **2**, **3**, **4**, **5**, and **6** correspond to (2*S*)-5,7,2′,6′-tetrahydroxy-6,8-di (γ,γ-dimethylallyl) flavanone, (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone), (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethylpyrano [5″, 6″: 6, 7]

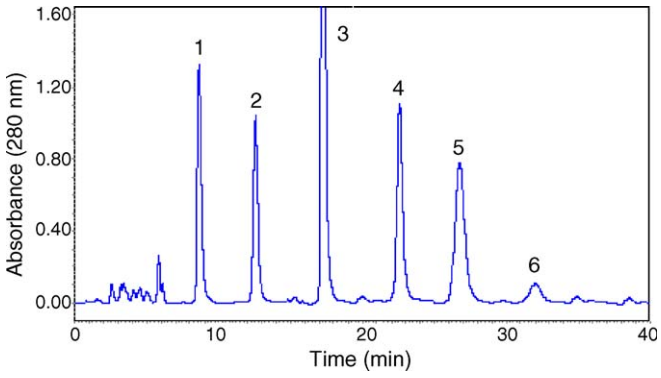

Fig. 2. HPLC chromatogram of the crude extract from the leaf of *P. villosa* after resin column chromatography. Column: reversed-phase Lichrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm); mobile phase: CH_3CN–MeOH–H_2O–HAC (40: 25: 35: 2, v/v); flow rate: 0.8 ml/min; UV wavelength: 280 nm; column temperature: 30 °C; peaks **1**, **2**, **3**, **4**, **5**, and **6** correspond to (2*S*)-5,7,2′,6′-tetrahydroxy-6,8-di (γ,γ-dimethylallyl) flavanone, (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone, (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone, (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethylpyrano [5″, 6″: 6, 7] flavanone, (2*S*, 3″*S*)-5,2′,6′-trihydroxy-3″-γ, γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydro-pyrano [5″, 6″: 6, 7] flavanone and licoagrochalcone B, respectively.

flavanone, (2*S*, 3″*S*)-5,2′,6′-trihy-droxy-3″-γ,γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydropyrano [5″, 6″: 6, 7] flavanone and licoagrochalcone B, and present the contents of 12.2%, 9.6%, 21.5%, 12.5%, 10.9% and 2.8%, respectively.

3.2. CCC isolation and separation

In CCC, the selection of two-phase solvent system is the most important for successful separation, and is also the most difficult step; it is estimated that about 90% of the entire work in CCC is spent on that. If only one component requires to be isolated from others, the standard CCC method, which uses a constant flow-rate of the mobile phase, could be used. In order to isolate more different compounds, step-wise elution or stepwise increasing the flow-rate of the mobile phase might be adopted [14–16]. First, CCC experiments were carried out with the two-phase solvent system composed of *n*-hexane–methanol at a volume ratio of 1: 1 (v/v), it was difficult to purify the target compounds from the crude extract, because their retention time was short. Subsequently, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 1: 1: 1: 1 (v/v) was tested. Although the peak resolution was improved, and peaks **1**, **2** and **3** could be isolated from others, it was difficult to separate other compounds. And then the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 10: 13: 13: 10 was used, the peaks **1**, **2**, **3** and **4** were purified and separated at the flow rate of 1.0 ml/min in less than 10 h, but the other two compounds were retained in the column for a long time (15 h) and more mobile phase was required. When the flow rate was increased to 2.0 ml/min, peaks **1** and **2** were combined together, while other compounds were obtained from other constituents in less 8 h. Finally, the method with stepwise increasing the flow-rate of the mobile phase was attempted with this two-phase solvent system. That is, the flow-rate of the mobile phase was kept at 1.0 ml/min before 110 min, and then increased to 2.0 ml/min after that moment.

At the same time, the influence of the separation temperature and the revolution speed were also investigated. The temperature has significant effect on K values, the retention of stationary phase and the mutual solvency of the two phases, which mainly due to the effects of the thermodynamics and the re-partition of the two phases in CCC column, and high temperature led to resist the loss of stationary phase and increase the retention of the stationary phase [17]. After tested at 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, it can be seen that good result can be obtained when the separation temperature was controlled at 35 °C. The revolution speed has a great influence to the retention of stationary phase, high rotary speed can increase the retention of the stationary phase. In our experiment, the revolution speed was set at 900 rpm.

Under the above optimized separation conditions, the isolation of the target compounds was achieved with good resolution and the retention of the stationary phase was satisfactory (67%), and the CCC separation time was approximately 400 min (CCC chromatogram is shown in Fig. 3). After the six compounds were eluted out, in order to save solvents and time, the remaining compounds in the column were removed by forcing out the

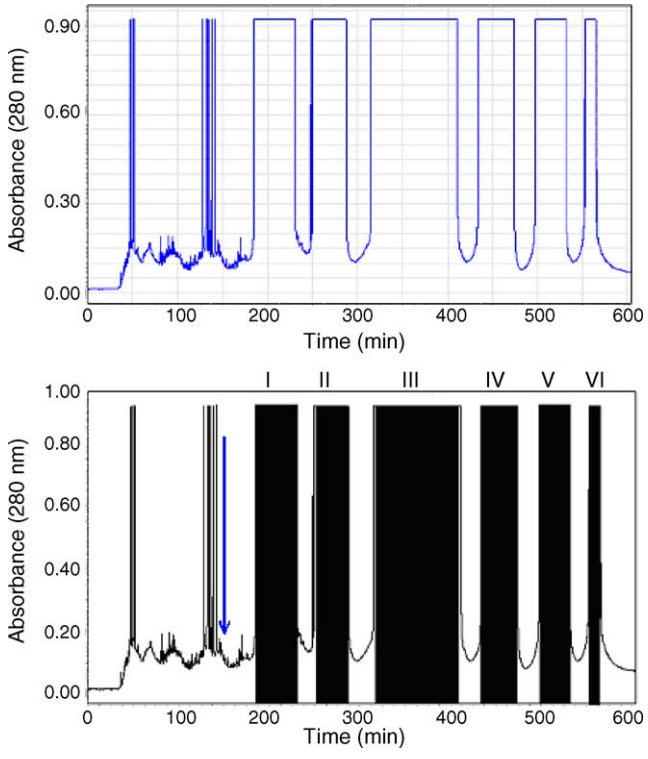

Fig. 3. CCC chromatogram of the crude extract from the leaf of *P. villosa* after cleaning-up by AB-8 macroporous resin. Solvent system: *n*-hexane–ethyl acetate–methanol–water (10: 13: 13: 10, v/v); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 900 rpm; separation temperature: 35 °C; sample size: 400 mg; retention of stationary phase: 67%; sample loop: 20 ml; detection wavelength: 280 nm. I, II, III, IV, V and VI are collected fractions. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 to 2.0 ml/min after 110 min.

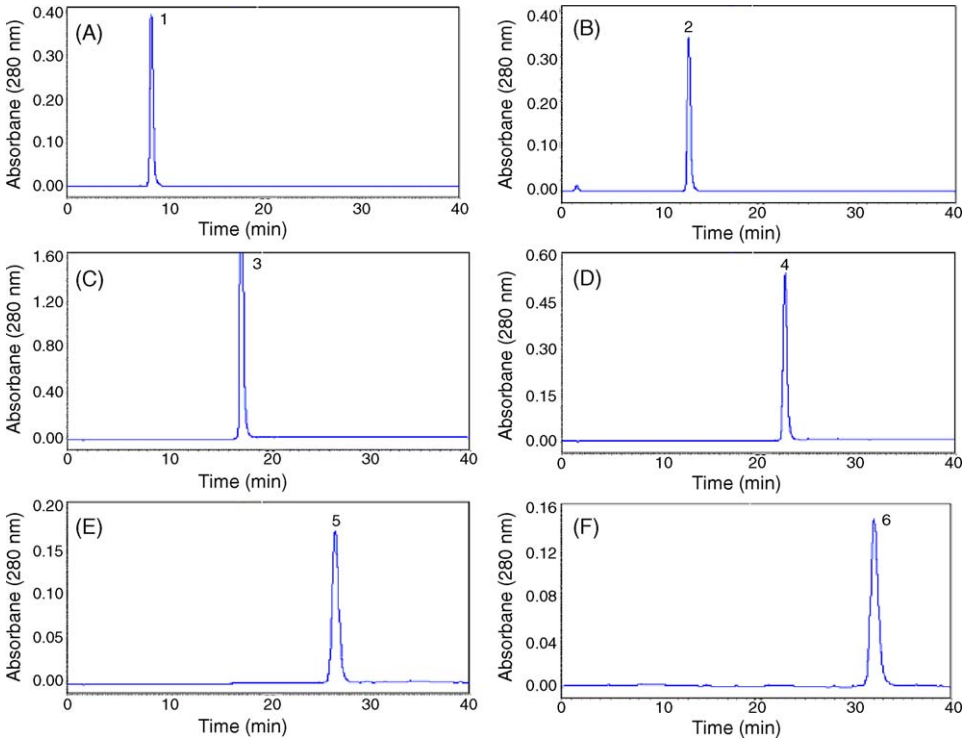

Fig. 4. HPLC chromatography of the fractions obtained by CCC. HPLC analytical conditions and the peaks are the same shown in Fig. 2. A: fraction I ; B: fraction II; C: fraction III; D: fraction IV; E: fraction V; F: fraction VI.

stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was not to be reused. Fig. 3 shows the preparative CCC isolation of 400 mg of crude extract using the solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ration of 10: 13: 13: 10 (v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 ml/min to 2.0 ml/min after 110 min. This separation yielded 44.9 mg compound **1** at 99.1% purity, 35.5 mg compound **2** at 98.8% purity, 79.8 mg compound **3** at 99.3% purity, 45.8 mg compound **4** at 98.8% purity, 39.8 mg compound **5** at 98.6% purity and 9.6 mg compound **6** at 97.5%, respectively, from 400 mg of the crude extract in one-step separation run (HPLC chromatograms are shown in Fig. 4). The recoveries of the six compounds were 91.2%, 91.4%, 92.1%, 90.5%, 90.3% and 89.7%, respectively, only in CCC run.

3.3. Structural elucidation of the isolated compounds

Compounds **1** and **6** (fraction I and VI) were two known compounds and have been isolated by us from *P. villosa* [7]. Their UV, IR, MS, ^{1}H NMR and ^{13}C NMR data are in agreement with (2*S*)-5,7,2′,6′-tetrahydroxy-6,8-di (γ,γ-dimethylallyl) flavanone and licoagrochalcone B in the literatures [7,18].

Compound **2** was isolated as light yellow powder. Its molecular formula was determined as $C_{25}H_{28}O_6$ by high resolution (HR)-electrospray ionization (ESI)-MS *m/z* 424.1562 (calcd. 424. 1234), ESI-MS *m/z* 423 $[M\text{-}H]^-$ and 847 $[2M\text{-}H]^-$, ^{13}C and DEPT NMR experiments showed 3 × CH_3, 4 × CH_2, 7 × CH, 11 × C. The IR spectrum (KBr) of **2** indicated the presence of hydroxyl (3434 cm^{-1}), conjugated carbonyl (1670 cm^{-1}), and aromatic (1600 and 1457 cm^{-1}) groups. The UV spectrum (MeOH, λ_{max}) exhibited absorption bands at 340, 293, 237 nm. The dihydroflavanone of compound **2** was deduced from the NMR spectra which showed an oxymethine, a carbonyl group and a methylene at δ_C 71.38, 197.49 and 39.03, respectively, and an ABX system [δ_H 2.47 (*dd*, *J* = 17.0, 3.0 Hz, H-3$_e$), 3.92 (*dd*, *J* = 17.0, 14.0 Hz, H-3$_a$) and 5.86 (*dd*, *J* = 14.0, 3.0 Hz, H-2), typically assignable to two H-3 and one H-2 protons of a flavanone]. The ^{1}H NMR signals at δ_H 6.39 (*d*, 2H, *J* = 8.0 Hz, H-3′, 5′) and 6.98 (t, 1H, *J* = 8.0 Hz, H-4′) were attributed for three adjacent protons on an aromatic ring. A lavandulyl group was deduced by the ^{1}H NMR signals at [δ_H 1.47, 1.51, 1.58 (s, 3H × 3), 1.96 (m, 2H), 2.46 (m, 2H), 2.45 (m, 1H), 4.56 (s, 1H), 4.53 (s, 1H), 4.93 (brs, 1H)] and ^{13}C NMR signals at [δ_C 26.48 (C-1″), 46.13 (C-2″), 147.85 (C-3″), 110.23 (C-4″), 18.40 (C-5″), 30.68 (C-1‴), 123.32 (C-2‴), 130.24 (C-3‴), 17.31 (C-4‴) and 25.20 (C-5‴)]. The point of attachment of the lavandulyl group was established unambiguously as C-6 by HMBC experiment. The coupling constant ($J_{2,3}$ = 14.0 Hz) between protons in the 2 and 3 positions, which was indicative of axial–axial coupling, revealed the C-2 hydrogen is axial and ring B equatorial [19]. The chiral centre of C-2 was assigned as S-configuration on the basis of its negative optical rotation, $[\alpha]_D$-150° (C. 1.00 MeOH) and equatorial B-ring [20]. All ^{1}H and ^{13}C NMR assignments (shown in Table 1) for compound **2** were performed by ^{1}H–^{1}H COSY, HMQC and HMBC experiments (main HMBC and NOESY correlations are shown in Fig. 5). Thus the compound **2** was determined to be (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone (shown in Fig. 1).

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **2** in [$^{2}H_6$] dimethyl sulfoxide (DMSO-d_6)

Position	δ_C	δ_H[a,b]	Position	δ_C	δ_H[a,b]
2	71.38	5.86 *dd* (14.0, 3.0)	3′,5′	106.71	6.39 *d* (8.0)
3	39.03	2.47 *dd* (17.0, 3.0) 3.92 *dd* (17.0, 14.0)	4′	129.43	6.98 *t* (8.0)
4	197.49	–	1″	26.48	2.46 *m*
5	161.27	12.30 *brs*	2″	46.13	2.45 *m*
6	106.19	–	3″	147.85	–
7	164.68		4″	110.23	4.56 *s* 4.53 *s*
8	94.81	5.97 *s*	5″	18.40	1.51 *s*
9	161.09	–	1‴	30.68	1.96 *m*
10	101.35	–	2‴	123.32	4.93 *brs*
1′	110.43	–	3‴	130.24	–
2′,6′	157.12	–	4‴	17.31	1.47 *s*
			5‴	25.20	1.58 *s*

[a] *J* in Hz.
[b] Signals were assigned by HMQC, HMBC and ^{1}H–^{1}H COSY experiments.

Compound **3** was isolated as white powder, UVλ_{max}^{MeOH}: 338, 290, 232 nm, IR (KBr) υ_{max}cm^{-1}: 3410 (OH), 1686 (C=O), 1623, 1469. ESI-MS: 423 [M-H]$^-$, 847 [2M-H]$^-$, HR-ESI-MS *m/z* 424.1246 for $C_{25}H_{28}O_6$ (calcd. 424.1234). This formula can be validated through ^{13}C NMR, ^{1}H NMR and DEPT spectra. The ^{1}H NMR signals at [δ_H 5.84 (*dd*, 1H, *J* = 14.0, 3.0 Hz, H-2), δ 2.42 (*dd*, 1H, *J* = 17.0, 3.0 Hz, H-3$_e$) and δ 3.94 (*dd*, 1H, *J* = 14.0, 17.0 Hz, H-3$_a$)], and ^{13}C NMR signals at [δ_C 71.50 (C-2), 39.65 (C-3) and 197.74 (C-4)] were characteristic of a dihydroflavanone for an ABX system. A lavandulyl group was deduced by the ^{1}H NMR signals at [δ_H 1.52, 1.60, 1.65 (s, 3H × 3), 2.02 (m, 2H), 2.50 (m, 2H), 2.47 (m, 1H), 4.59 (s, 1H), 4.53 (s, 1H), 4.99 (brs, 1H)] and ^{13}C NMR signals at [δ_C 26.23 (C-1″), 46.09 (C-2″), 147.73 (C-3″), 110.70 (C-4″), 18.47 (C-5″), 30.89 (C-1‴), 123.58 (C-2‴), 130.45 (C-3‴), 17.57 (C-4‴) and 25.44 (C-5‴)]. The ^{1}H NMR signals at δ_H 5.89 (2H, s, H-3′, 5′) and ^{13}C NMR signals at [δ_c 157.96 (C-2′, 6′), 106.4(C-3′, 5′) and 158.79 (C-4′)] indicated two hydroxyl group and two protons on B-ring, and the point of attachment of the lavandulyl group was established unambiguously as C-4′ by the ^{1}H-^{13}C long-range coupling and HMBC experiments. The ^{1}H NMR signals at δ_H 5.68 (1H, s, H-6) and 5.87 (1H, s, H-8) and ^{13}C NMR signals at δ_c 94.03 (C-6) and 94.18 (C-8) indicated that there have substitutes on C-6 and C-8. The absolute stereochemistry at C-2 of compound **3** was established as S likely to the compound **2**. All ^{1}H and ^{13}C NMR assignments (shown in Table 2) for compound **3** were performed by ^{1}H–^{1}H COSY, HMQC and HMBC experiments (main HMBC and NOESY correlations are shown in Fig. 5). Thus the compound **3** was determined to be (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone (shown in Fig. 1).

Compound **4** was isolated as light yellow powder, mp: 167∼168 °C. The ESI-MS of **4** showed a molecular [M-H]$^-$

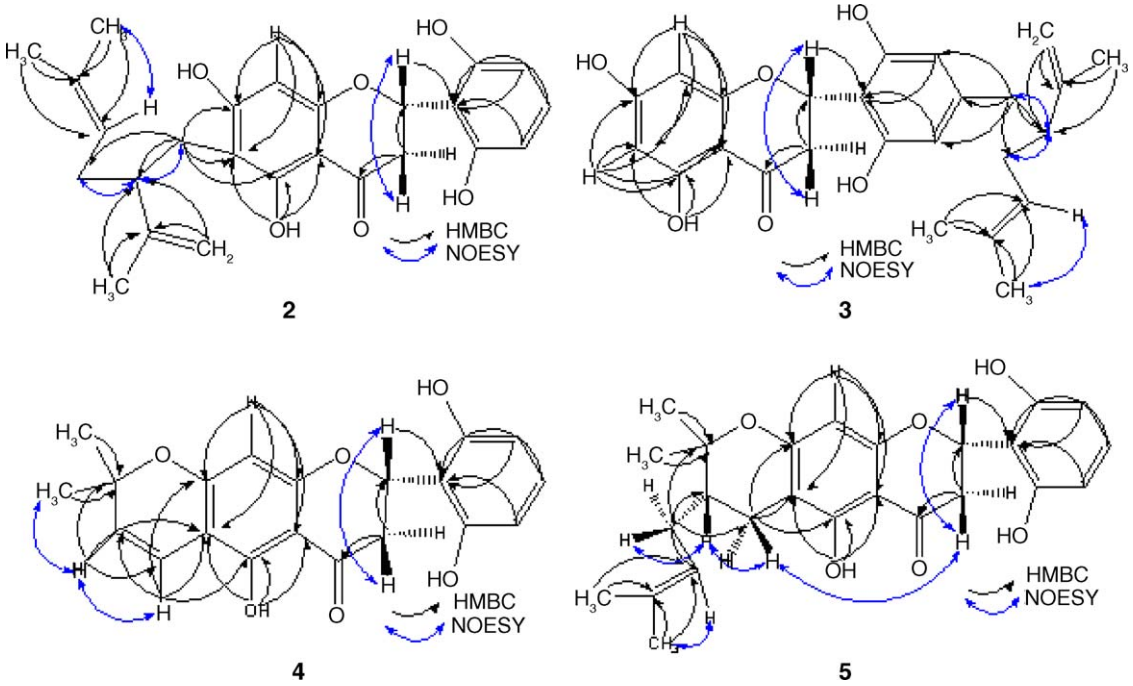

Fig. 5. The main HMBC and NOESY corrections of the four new compounds.

Table 2
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **3** in DMSO-d_6

Position	δ_C	δ_H[a,b]	Position	δ_C	δ_H[a,b]
2	71.50	5.84 *dd* (14.0, 3.0)	3′,5′	106.40	5.89 *s*
3	39.65	2.42 *dd* (17.0, 3.0) 3.94 *dd* (17.0, 14.0)	4′	158.79	–
4	197.74	–	1″	26.23	2.50 *m*
5	161.77	12.56 *brs*	2″	46.09	2.47 *m*
6	94.03	5.68 *s*	3″	147.73	–
7	164.89		4″	110.70	4.59 *s* 4.53 *s*
8	94.58	5.87 *s*	5″	18.47	1.60 *s*
9	161.33	–	1‴	30.89	2.02 *m*
10	101.08	–	2‴	123.58	4.99 *brs*
1′	110.70	–	3‴	130.45	–
2′,6′	157.96	–	4‴	17.57	1.52 *s*
			5‴	25.44	1.65 *s*

[a] *J* in Hz.
[b] Signals were assigned by HMQC, HMBC and ^{1}H–^{1}H COSY experiments.

ion at *m/z* 353 and the molecular formula of $C_{20}H_{18}O_6$ was derived from the HR-ESI-MS (*m/z* 354.1126 calcd. 354.1131). This formula can also be validated through ^{13}C NMR, ^{1}H NMR and DEPT spectra. The IR spectrum (KBr) of **4** indicated the presence of hydroxyl (3412 cm^{-1}), conjugated carbonyl (1668 cm^{-1}), and aromatic (1587 and 1465 cm^{-1}) groups. The UV spectrum (MeOH, λ_{max}) of **4** exhibited maxima at 275 and 310 nm. The ^{1}H NMR spectrum of **4** (Table 3) showed resonances for an ABX system at δ_H 2.48 (*dd*, 1H, *J* = 3.0, 17.0 Hz), δ_H 3.90 (*dd*, 1H, *J* = 13.0, 17.0 Hz) and δ_H 5.78 (*dd*, 1H, *J* = 3.0, 13.0 Hz), which is diagnostic for H-2 and H-3 of a flavanone nucleus. The ^{1}H NMR signals at δ_H 6.37 (*d*, 2H, *J* = 8.0 Hz, H-3′, 5′) and 6.99 (t, 1H, *J* = 8.0 Hz, H-4′) were attributed for three adjacent protons on an aromatic ring, and δ_H 12.28 was characteristic of 5-OH. The dimethylchromene ring was elucidated by the ^{1}H NMR signals at [δ_H 1.36, 1.39 (s, each 3H, Me × 2), 5.94, 6.53 (*d*, each 1H, *J* = 10.0 Hz, *cis*-olefinic proton)] and ^{13}C NMR signals [δ_c 27.69, 27.98 (Me × 2), 77.88 (C-2″), two *cis*-olefinic carbons at 126.37 (C-3″) and 115.02 (C-4″)]. These data suggested that compound **4** has a flavanone skeleton with one hydroxyl group and a dimethylpyran moiety, and these inferences were confirmed using the DEPT, ^{1}H–^{1}H COSY and HMQC NMR techniques. The presences of the substituents at positions C-5 (hydroxyl group), C-6 and C-7 (dimethylpyran) were deduced using the HMBC experiment (Fig. 5). The coupling constant ($J_{2,3}$ = 13.0 Hz) between protons in the 2 and 3 positions, which was indicative of axial–axial coupling, revealed the C-2 hydrogen is axial and ring B equatorial. The chiral centre of C-2 was assigned as S-configuration on the basis of its negative optical rotation, $[\alpha]_D$-100° (C. 1.00 MeOH) and equatorial B-ring. All ^{1}H and ^{13}C NMR assignments for compound **4** are shown in Table 3. The chemical structure, main HMBC and NOESY correlations are shown in Figs. 1 and 5. Thus the compound **4** was determined to be (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethylpyrano [5″, 6″: 6, 7] flavanone.

Compound **5** was isolated as white powder. Its molecular formula was determined as $C_{25}H_{28}O_6$ by HR-ESI-MS *m/z* 424.1262 (calcd. 424.1234), ESI-MS *m/z* 423 [M-H]$^-$ and 847 [2M-H]$^-$, ^{13}C and DEPT NMR experiments showed 4 × CH_3, 3 × CH_2, 7 × CH, 11 × C. The IR spectrum (KBr) of **5** indicated the presence of hydroxyl (3400 cm^{-1}), conjugated carbonyl (1665 cm^{-1}), and aromatic (1568 and 1443 cm^{-1}) groups. The UV spectrum (MeOH, λ_{max}) exhibited absorption bands at 330 and 291 nm. The dihydroflavanone of compound **5** was deduced from the NMR spectra which showed an oxymethine, a carbonyl group and a methylene at δ_C 71.94, 197.84 and 39.04, respectively, and an ABX system [δ_H 2.46 (*dd*, *J* = 17.0, 3.0 Hz, H-3$_e$), 3.89 (*dd*, *J* = 17.0, 13.0 Hz, H-3$_a$) and 5.80 (*dd*, *J* = 13.0,

Table 3
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **4** in DMSO-d_6

Position	δ_C	δ_H[a,b]	Position	δ_C	δ_H[a,b]
2	72.00	5.78 *dd* (3.0, 13.0)	1′	109.96	–
3	39.00	2.48 *dd* (3.0, 17.0) 3.90 *dd* (13.0, 17.0)	2′,6′	157.34	–
4	197.62	–	3′,5′	106.84	6.37 *d* (8.0)
5	159.52	12.28 *brs*	4′	129.89	6.99 *t* (8.0)
6	106.78	–	2″	77.88	–
7	162.25		3″	126.37	5.94 *d* (10.0)
8	96.01	5.90 *s*	4″	115.02	6.35 *d* (10.0)
9	157.86	–	2″-Me	27.69	1.36 *s*
10	100.98	–	2″-Me	27.98	1.39 *s*

[a] *J* in Hz.
[b] Signals were assigned by HMQC, HMBC and ^{1}H–^{1}H COSY experiments.

Table 4
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **5** in DMSO-d_6

Position	δ_C	δ_H[a,b]	Position	δ_C	δ_H[a,b]
2	71.94	5.80 *dd* (14.0, 3.0)	3′,5′	106.90	6.39 *d* (8.0)
3	39.04	2.46 *dd* (17.0, 3.0)	4′	129.92	6.98 *t* (8.0)
		3.89 *dd* (17.0, 14.0)			
4	197.84	–	2″	79.15	–
5	160.93	12.18 *brs*	3″	40.16	2.18 *m*
6	101.92	–	4″	21.53	2.20 *dd* (16.0, 12.0)
					2.63 *dd* (12.0, 3.0)
7	161.43		2″-Me	20.89	1.16 *s*
8	95.65	5.89 *s*	2″-Me	25.48	1.36 *s*
9	160.84	–	1‴	28.68	2.02 *ddd* (16.5, 12.2, 7.5)
					2.11 *ddd* (12.2, 7.8, 3.0)
10	100.34	–	2‴	122.51	5.12 *brs*
1′	110.20	–	3‴	132.42	–
2′,6′	157.35	–	4‴	17.56	1.51 *s*
			5‴	27.20	1.64 *s*

[a] *J* in Hz.
[b] Signals were assigned by HMQC, HMBC and ^{1}H–^{1}H COSY experiments.

3.0 Hz, H-2), typically assignable to H-3 and H-2 protons of a flavanone]. The ^{1}H NMR signals at δ_H 1.51, 1.64 (1H each, s, Me), δ_H 5.12 (1H, m, olefinic proton, H-2‴), δ_H 2.02, 2.11 (1H each, *dd*, *J* = 8.0, 8.0 Hz, H-1‴) and ^{13}C NMR signals at [δ_C 17.56, 27.20 (Me × 2), 28.68 (C-1‴), 122.51 (C-2‴), 132.42 (C-3‴) indicated a dimethylallyl group was existed in the compound **5** and δ_H 12.18 was characteristic of 5-OH. The dihydro-dimethylchromene ring was elucidated by the ^{1}H NMR signals [δ_H 1.16, 1.36 (3H each, s, Me × 2), 2.18 (1H, m, H-3″), 2.20, 2.63 (1H each, *dd*, *J* = 10.0, 10.0 Hz, H-4″)] and ^{13}C NMR signals [δ_c 20.89, 25.48 (Me × 2), 79.15 (C-2″), 40.16 (C-3″), 21.53 (C-4″). Comparison with the dimethylchromene ring, no *cis*-olefinic protons and carbons were observed. These data suggested that compound **5** has a flavanone skeleton with one hydroxyl group and a dihydro-dimethylpyran moiety, and these inferences were confirmed using the DEPT, ^{1}H-^{1}H COSY and HMQC NMR techniques. The position of the substituents were deduced as accruing at C-5 and C-6, C-7 (dihydro-dimethylpyran) using the HMBC experiment. The absolute stereochemistry at C-2 of compound **5** was established as S based on NOESY technique. All ^{1}H and ^{13}C NMR assignments for compound **5** were shown in Table 4. The main HMBC and NOESY correlations are shown in Fig. 5. Thus the compound **5** was determined to be (2*S*, 3″*S*)-5,2′,6′-trihydroxy-3″-γ,γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydropyrano [5″, 6″: 6, 7] flavanone (shown in Fig. 1).

3.4. Anticancer activity assay of the isolated compounds

In anticancer assay, seven kinds of typical human cancer cell lines were selected to evaluate the compounds' inhibition activities to cancer cell growth including human lung cancer cell A549, human liver cancer cell BEL-7402, human esophageal cancer cell HT-29, human breast cancer sell MCF-7, human gastric cancer cell SGC-7901, human leukemia cancer cell K562 and human kidney cancer cell A498. The data are reported Table 5, and the results (IC_{50} < 7 μg/ml) indicated that the compounds **1**, **2** and **3** exhibited high inhibition effect (IC_{50} < 10 μg/ml) to tumor cells' growth in a dose-dependent manner, and when the concentration of the compounds was over 15 μg/ml, the cancer cells were totally inhibited, especially to K562 cancer cell (IC_{50} < 3.1 μg/ml). The compounds **4**, **5** and **6** could also inhibit the cancer cells' growth, but the inhibition effect was weaker than the compounds **1**, **2** and **3** (IC_{50} < 30 μg/ml).

Table 5
The IC_{50} values of the isolated compounds to inhibit human cancer cells growth by MTT method

Human cancer cells	Compounds IC_{50} (μg/ml)[a]					
	1	**2**	**3**	**4**	**5**	**6**
A549	3.93	4.87	4.02	15.21	9.68	10.25
BEL-7402	4.60	3.64	2.85	13.20	7.52	9.98
HT-29	4.08	5.28	4.26	20.22	14.36	25.36
MCF-7	5.72	7.94	7.62	35.48	18.53	28.54
SGC-7901	6.59	10.20	9.25	49.52	30.21	38.45
K562	1.64	2.42	3.04	12.25	6.58	12.23
A498	4.52	8.85	7.92	46.24	25.46	35.21

[a] IC_{50} was expressed as the drug concentration resulting in a 50% inhibition of cell growth and calculated from dose–inhibition curves.

4. Conclusion

Six flavanoids including four novel ones were successfully isolated and separated by high-speed counter-current chromatography from the leaf of *P. villosa*. Our research demonstrated that CCC is a powerful technique to separate and isolate chemical constituents from medicinal plants, and the pharmacology test showed that the compounds **1**, **2** and **3** exhibited high anticancer activities. In the light of these results, in vivo actions and clinical applications of the compounds are required further investigation.

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