Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1092 (2005) 235–240
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Preparative isolation and separation of a novel and two known flavonoids from *Patrinia villosa* Juss by high-speed counter-current chromatography

Jinyong Peng [a], Genjin Yang [b], Guorong Fan [a,*], Yutian Wu [a]

[a] *Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, China*
[b] *Pharmaceutical Analysis and Test Center, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, China*

Received 21 May 2005; received in revised form 8 July 2005; accepted 11 July 2005
Available online 15 August 2005

Abstract

Preparative high-speed counter-current chromatography (HSCCC) was successfully used for isolation and separation three flavonoids including bolusanthol B, a novel compound named 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and tetrapterol I from *Patrinia villosa* Juss using two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratio of 10:11:11:8 (v/v). A total of 25.4 mg bolusanthol B, 52.5 mg 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and 50.2 mg tetrapterol I were obtained from 250 mg crude extract with purities of 96.8%, 99.2% and 99.3%, respectively determined by HPLC in one single operation and less than 5 h. The structure identification was performed by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR. Among then, bolusanthol B and tetrapterol I were obtained from the plant of *Patrinia* genius for the first time, and 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone was a novel prenylated flavonoid and discovered from nature for the first time.

Keywords: *Patrinia villosa* Juss; Flavonoid; Preparative chromatography; Counter-current chromatography; Prenylated 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 growing in China. *Patrinia* species have been used as medicinal plants for more than 2000 years from *ShenNong-BenCaoJing*, a famous ancient Chinese medicinal literary, and some of them still used in folk medicine as anti-virus and -bacteria [1,2], especially two species, *P. scabiosaefolia* Fisch and *P. villosa* Juss (*BaiJiangCao* in Chinese).

With regard to the chemical constituents of this genus, we have found more research about *P. scabiosaefolia* Fisch [3], *P. scabra* [4] and *P. gibbosa* [5] than *P. villosa* Juss. Except for some iridoids [6,7], we have isolated and separated two C-glycosylflavones (isovitexin and isoorientin) [8] and a peptide derivative aurentiamide acetate [9] from it. A literature search did not yield any more references to early report on study of chemicals from the medicinal herb *P. villosa* Juss. So, further chemical research and discovery from *P. villosa* Juss is warranted for exploitation new TCM products and pharmacological tests. However, pure products are often isolated and separated by some conventional methods including silica gel, polyamide and preparative high-performance liquid chromatography (HPLC), which are tedious, time consuming, requiring multiple chromatographic steps. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support [10], has an excellent sample recovery. So, it has been successfully applied to isolate and purify a number of natural products [11–15].

* Corresponding author. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: Guorfan@yahoo.com.cn (G. Fan).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.07.073

The aim of the present paper, therefore, was that a preparative high-speed counter-current chromatography was used as a tool to separate and purify chemicals from *P. villosa* Juss and an efficient method was developed for the isolation and separation of bolusanthol B, 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and tetrapterol I with high purities from *P. villosa* Juss by HSCCC. As far as we know, bolusanthol B and tetrapterol I were obtained from the plants of *P. genius* for the first time, and 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone was a novel prenylated flavonoid and discovered from nature for the first time.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a model TBE–300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus 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 HSCCC 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).

The analytical HPLC system used throughout this study consisted of LC–10AT pump and a SPD–10A UV–vis detector (Shimadzu, Japan), and a model N2000 workstation (Zhejiang University, Hangzhou, China). D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China).

2.2. Reagents

Petroleum ether, ethyl acetate, *n*-hexane, methanol, ethanol, acetic acid were analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The *P. villosa* Juss was purchased from a local drug store (Shanghai, China).

2.3. Preparation of the crude extract

The *P. villosa* Juss was ground into powder, 8.0 kg of the powder was extracted by reflux with 8.0×10^4 ml 70% aqueous ethanol for two times. The mixture was filtered, and 6.1×10^4 ml filtrate was collected. The extract was then concentrated to no ethanol by rotary vaporization at 60 °C under reduced pressure and 2000 ml residue was obtained. Then the residue was redissolved in water (total volume 4000 ml), which was added into a glass column (6.0 cm × 80 cm, contained 3.0 kg D101 macroporous resin). 1.2×10^4 ml water was first used to elute the resin until the elution was nearly no color, and 8000 ml 40% aqueous ethanol was used to elute the resin, too. Then 1.0×10^4 ml 85% aqueous ethanol was used to elute the target compounds, and 20 elution fractions (500 ml for each) were collected and three (from 8 to 10 fraction) were united and evaporated to dryness according to HPLC analysis, which was used for HSCCC isolation and separation.

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

Two-phase solvent system was used in the present study, *n*-hexane–ethyl acetate–methanol–water (10:11:11:8, v/v) was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the sample in the 10 ml lower phase of solvent system for isolation and purification.

2.5. HSCCC separation procedure

In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.8 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml sample solution containing 250 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV–vis detector at 280 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis and identification of CCC peak fractions

The crude sample and the peak fraction obtained by HSCCC were analyzed by high-performance liquid chromatography. The column used was a Lichrospher C_{18} (6.0 mm × 150 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–H_2O–HAC (50:50:1, v/v/v). The flow rate was 0.8 ml/min, and the effluent was monitored at 280 nm (Fig. 1).

Identification of the CCC peak fractions was carried out by UV (Cary-50, Varian), IR (Hitachi 275-50), MS (Finnigan MAT 711), ^{1}H NMR, ^{13}C NMR and 2D NMR spectra (Varian Unity Inova–500).

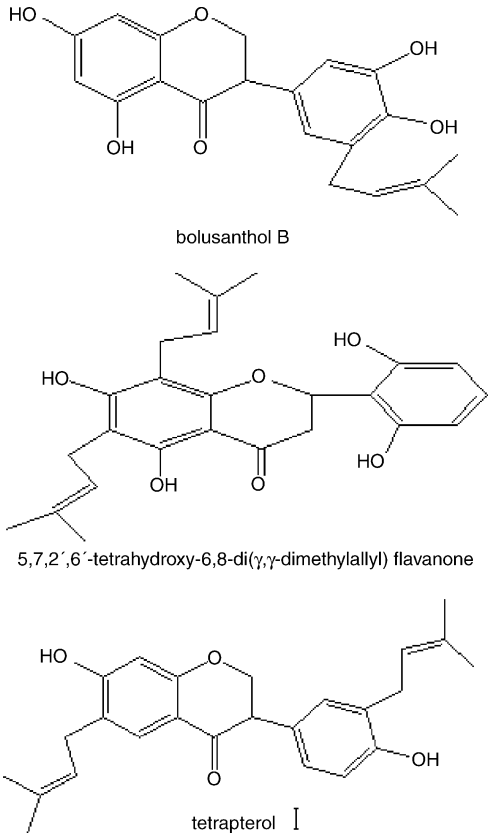

Fig. 1. The chemical structures of bolusanthol B, 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and tetrapterol I.

3. Results and discussion

3.1. Optimization of HPLC method

First, the crude extract used for further HSCCC isolation was analyzed by HPLC. So, a good HPLC condition was required. In our research, different mobile phases (methanol–water, acetonitrile–water) with different concentration of acetic acid, different flow rates and column temperature were all tested. The result indicated that the mobile phase was acetonitrile–water–acetic acid at a volume ratio of 50:50:1 (v/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. It is obvious that the crude extract mainly contained three peaks. Peaks 1–3 correspond to bolusanthol B, 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and tetrapterol I, respectively.

3.2. Selection of two-phase solvent system and other conditions of HSCCC

In HSCCC, a suitable two-phase solvent system was critical for a successful isolation and separation. In our

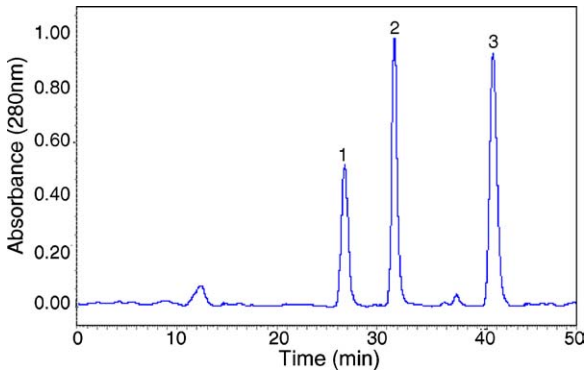

Fig. 2. HPLC chromatography of the crude extract from *P. villosa* Juss. Column: reversed-phase Lichrospher C_{18} (6.0 mm × 150 mm i.d., 5 μm); mobile phase: CH_3CN–H_2O–HAC (50:50:1, v/v/v); flow rate: 0.8 ml/min; UV wavelength: 280 nm; column temperature: 30 °C; (1): bolusanthol B; (2): 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone; and (3): tetrapterol I.

experiment, several kinds of solvent systems including *n*-hexane–ethyl acetate–methanol–water at different volume ratios (10:5:5:8, 10:7:7:8, 10:9:9:8, 10:11:11:8, 10:13:13:8, v/v) were tested. After trying the five kinds of solvent systems, we have found that the compounds in solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratios of 10:5:5:8, 10:7:7:8 and 10:9:9:8 (v/v) had large *K* values (Table 1), and small *K* values could be produced in solvent system composed of *n*-hexane–ethyl acetate–methanol–water (10:13:13:8, v/v). At last, the solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratio of 10:11:11:8 (v/v) was selected in the present paper, which was most suitable for our isolation and separation than others.

The influence of flow rate of mobile phase, the separation temperature and the revolution speed were also investigated. The result indicated that slow flow speed can produce a good separation, but more time and more mobile phase will be

Table 1
The partition coefficients (*K*) of the three compounds in different solvent systems

n-Hexane–ethyl acetate–methanol–water	Compound 1	Compound 2	Compound 3
10:5:5:8 (v/v)	2.68	3.06	4.33
10:7:7:8 (v/v)	1.88	2.40	3.86
10:9:9:8 (v/v)	1.41	1.74	3.20
10:11:11:8 (v/v)	0.61	1.10	2.59
10:13:13:8 (v/v)	0.26	0.75	1.64

Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 2 mg of crude sample placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase. Compounds 1–3 correspond to bolusanthol B, 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and tetrapterol I, respectively.

needed, and the chromatogram peak was extended. Considering these aspects, the flow tare was selected 1.8 ml/min in the present study. The temperature has significant effect on partition coefficient (K) values, the retention of stationary phase and the mutual solvency of the two phases. After tested at 15, 20, 25, 30, 35 and 40 °C, it can be seen that good result could be obtained when the separation temperature was controlled at 30 °C. The revolution speed has a great influence to the retention of stationary phase, high rotary speed can increased the retention of the stationary phase. In our experiment, the revolution speed was set at 800 rpm.

Under the optimized conditions, four fractions (I–III) were obtained in one-step elution and less than 5 h (HSCCC chromatogram is shown in Fig. 3), and the retention of the stationary phase was 63%. The obtained fractions produced 25.4 mg bolusanthol B, 52.5 mg 5,7, 2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone and 50.2 mg tetrapterol I in one single operation from 250 mg crude extract with purities of 96.8%, 99.2% and 99.3%, respectively determined by HPLC. The HPLC chromatograms of the fractions obtained by HSCCC are shown in Fig. 4. As expected, the HPLC analysis of each fraction revealed that the components eluted in the order of peaks 1 (bolusanthol B), 2 (5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone) and 3 (tetrapterol I).

3.3. The structural identification

The identification of the obtained materials was carried out by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR spectra as follows.

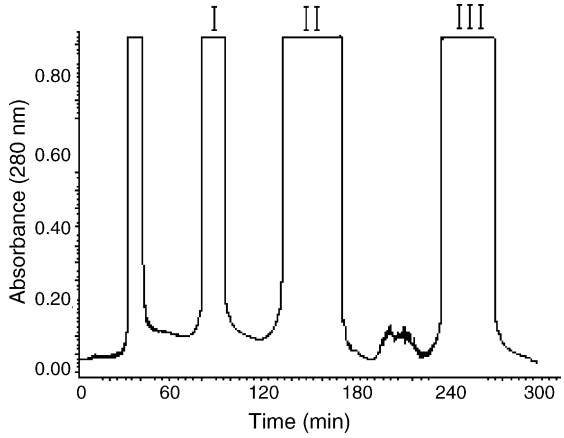

Fig. 3. HSCCC chromatogram of the crude extract from *P. villosa* Juss. Solvent system: *n*-hexane–ethyl acetate–methanol–water (10:11:11:8, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.8 ml/min; revolution speed: 800 rpm; separation temperature: 30 °C; sample size: 250 mg; retention of stationary phase: 63%; sample loop: 20 ml; detection wavelength: 280 nm.

Fractions I and III were known compounds, their UV, IR, MS, ^{1}H NMR and ^{13}C NMR data are in agreement with bolusanthol B and tetrapterol I in the literatures [16,17].

Fraction II: white powder, UV λ_{max}^{MeOH}: 337, 292, and 221 nm. m.p.: 185–187 °C. IR (KBr) υ_{max} cm^{-1}: 3416 (OH), 1690 (C=O), 1625, 1475, 1376, 1250, and 1130. ESI-MS: 423 $[M-H]^-$, 847 $[2M-H]^-$. HR-ESI-MS m/z 424.1236 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

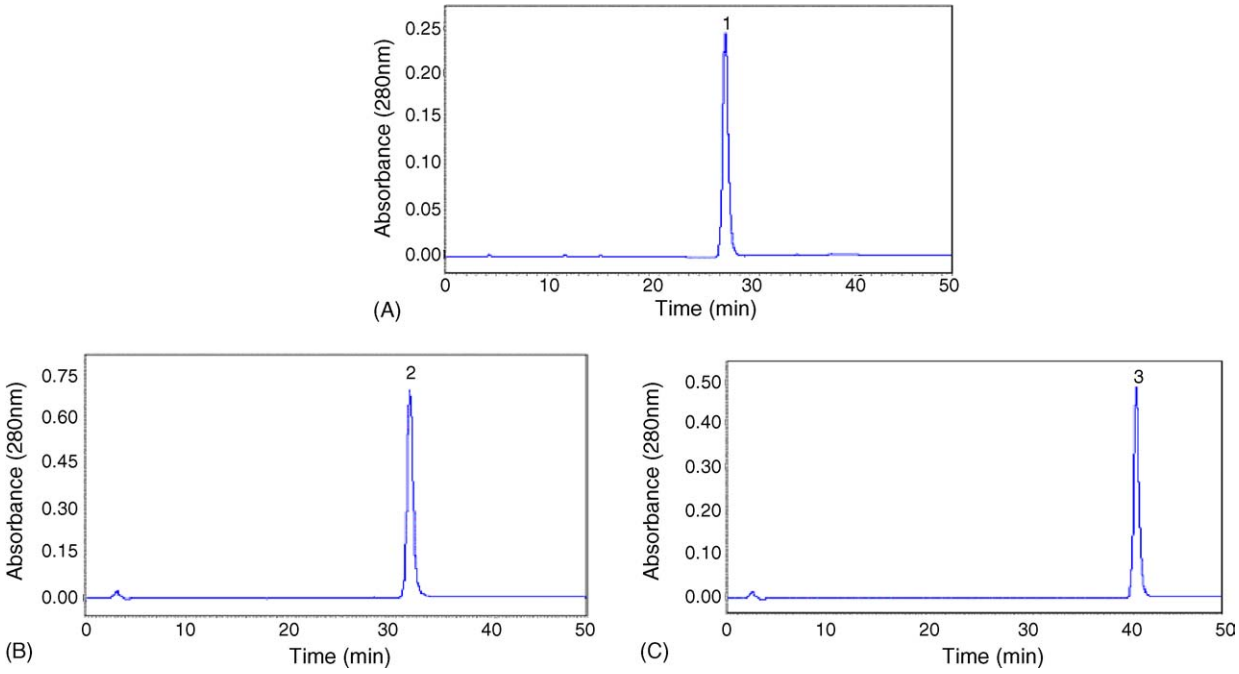

Fig. 4. HPLC chromatography of the fractions obtained by HSCCC. HPLC analysis conditions and the peaks are the same shown in Fig. 2. (A): Fraction I; (B): fraction II; and (C): fraction III.

Table 2
The ^{1}H NMR and ^{13}C NMR data of 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone in DMSO[a]

Position	δ_C	δ_H	Position	δ_C	δ_H
2	71.49	5.803 d (13.0)	3′, 5′	106.81	6.346 d (8.0)
3	39.27	2.438 d (17.0) 3.893 dd (13.0, 17.0)	4′	129.50	6.933 dd (8.0, 9.0)
4	197.74	–	1″	21.48	3.123 d (7.0)
5	158.68	12.468 brs	2″	122.96	5.111 dd (6.0, 7.0)
6	107.58	–	3″	129.97	–
7	161.70		4″	17.52	1.696 s
8	107.03	–	5″	25.33	1.604 s
9	157.20	–	1‴	20.87	3.112 dd (8.0, 8.0)
10	101.59	–	2‴	122.63	5.064 dd (7.0, 7.0)
1′	110.63	–	3‴	129.93	–
2′, 6′	158.55	–	4‴	17.16	1.548 s
			5‴	25.26	1.472 s

[a] TMS was used as internal standard; chemical shifts are shown in the δ scale with J values (Hz) in parentheses. Run at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR.

signals at [δ_H 5.803 (1H, d, J = 13.0 Hz, H-2), δ2.438 (1H, d, J = 17.0 Hz, H-3) and δ3.893 (1H, dd, J = 13.0, 17.0 Hz, H-3)], and ^{13}C NMR signals at [δ_C 71.49 (C-2), 39.27 (C-3) and 197.74 (C-4)] were characteristic of an flavanone. The signals at δ_H 6.346 (2H, d, J = 8.0 Hz) and 6.933 (1H, dd, J = 8.0, 9.0 Hz) for three adjacent protons on an aromatic nucleus. The presence of prenyl groups were confirmed from the ^{1}H NMR signals [(δ_H 3.123 (2H, d, J = 7.0 Hz, H-1″), 3.112 (2H, dd, J = 8.0, 8.0 Hz, H-1‴), 5.111 (1H, dd, J = 6.0, 7.0 Hz, H-2″), 5.064 (1H, dd, J = 7.0, 7.0 Hz, H-2″), 1.696 (3H, s, H-4″), 1.604 (3H, s, H-5″), 1.548 (3H, s, H-4‴), 1.472 (3H, s, H-5‴)), in which correlations were observed among allylic methylene signals [δ_H 3.123 (H-1″) and 3.112 (H-1‴)] and oxygenated carbon signals at [δ_C 118.68 (C-5) and 161.70 (C-7)], and carbon signals at [δ_C 161.70 (C-7) and 157.20 (C-9)] indicating the presence of prenyl moieties at positions C-6 and C-8. All ^{1}H NMR and ^{13}C NMR assignments (shown in Table 2) for fraction III were performed by COSY, HMQC and HMBC experiments (main HMBC correlations is shown in Fig. 5). Thus, fraction III was determined to be 5,7,2′,6′-tetrahydroxy -6,8-di(γ,γ-dimethylallyl) flavanone, a novel compound.

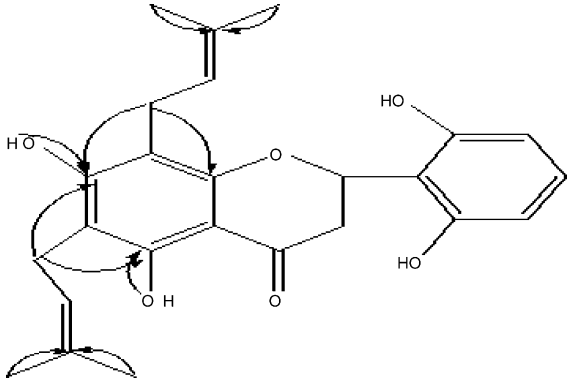

Fig. 5. The main HMBC correlations of 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone.

4. Conclusion

Three compounds including bolusanthol B, 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethyl-allyl) flavanone and tetrapterol I were successfully isolated and separated by high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratio of 10:11:11:8 (v/v) from the medicinal plant *P. villosa* Juss. Among then bolusanthol B and tetrapterol I were obtained from the plant of *Patrinia* genius for the first time, and 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone was a novel prenylated flavonoid and discovered from nature for the first time.

Acknowledgement

Financial support from Ministry of Science and Technology of China (863 project) is gratefully acknowledged.

References

[1] W.R. Jian, S.B. Ming, J. Chuangchun College Trad. Chin. Med. 13 (1997) 46.
[2] F.J. He, J.P. Yang, Y.J. Tian, Gan Su Med. 12 (1993) 161.
[3] L.T. Fang, L.F. Chun, T.Y. Ping, W.J. Hua, Nat. Prod. Res. Dev. 13 (2000) 71.
[4] Tanaka, N. Marubayashi, D.N. Yang, Phytochemistry 37 (1994) 467.
[5] S.U. Xie, S.H. Znouye, T. Shingu, M. Znouet, M. Doi, Phytochemistry 26 (1987) 561.
[6] C.J. Xu, X.Y. Zeng, D.Q. Yu, Acta Pharm. Sinica 20 (1985) 652.
[7] H. Taguchi, et al., Yakuyaka Zassshi 93 (1973) 607.
[8] J.Y. Peng, G.R. Fan, Z.Y. Hong, Y.F. Chai, Y.T. Wu, J. Chromatogr. A 1074 (2005) 111.
[9] J.Y. Peng, G.R. Fan, Y.T. Wu, J. Chromatogr. A 1083 (2005) 52.
[10] Y. Ito, CRC Crit. Rev, Anal. Chem. 17 (1986) 65.
[11] X. Wang, Y.Q. Wang, Y.L. Geng, F.W. Li, C.C. Zheng, J. Chromatogr. A 1036 (2004) 171.

[12] Y. Jiang, H.T. Lu, F. Chen, J. Chromatogr. A 1033 (2004) 183.
[13] Y. Wei, T.Y. Zhang, G.Q. Xu, Y. Ito, J. Chromatogr. A 929 (2001) 169.
[14] L. Lei, F.Q. Yang, T.Y. Zhang, P.F. Tu, L.J. Wu, Y. Ito, J. Chromatogr. A 912 (2001) 181.
[15] L. Chen, Y.S. Han, F.Q. Yang, T.Y. Zhang, J. Chromatogr. A 907 (2001) 343.
[16] G. Bojase, C.C.W. Wanjala, R.R.T. Majinda, Phytochemistry 56 (2001) 837.
[17] Y. Shirataki, S. Matsuoka, M. Komatsu, M. Ohyama, T. Tanaka, M. Iinuma, Phytochemistry 50 (1999) 695.