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Preparative Isolation and Purification of Three Stilbene Glycosides from the Tibetan Medicinal Plant *Rheum tanguticum* Maxim. Ex Balf. by High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – Stilbene glycosides are the primary constituents of *Rheum tanguticum* Maxim. ex Balf., to which different bioactivities has been attributed, including: anti-HIV, anti-oxidant, anti-tumour, anti-malarial, and anti-allergy activity. However, effective methods for the isolation and purification of stilbene glycosides, such as *trans*-rhapontin, *cis*-rhapontin and *trans*-desoxyrhaponticin, from this herb are not currently available.

Objective – To develop an efficient method for the preparative isolation and purification of three stilbene glycosides from *Rheum tanguticum* Maxim. ex Balf. via high-speed counter-current chromatography (HSCCC).

Methods – A solvent system composed of chloroform:*n*-butanol:methanol:water (4:1:3:2, v/v/v/v) was developed for the separation. The upper phase was used as the stationary phase, and the lower phase was used as the mobile phase. The flow rate was 1.8 mL/min. The apparatus was controlled at 800 rpm and 25 °C, and the effluent was monitored at 280 nm. Chemical constituents were analysed by high-performance liquid chromatography (HPLC), and their structures were identified by ^{1}H- and ^{13}C-NMR.

Results – Under the optimised conditions, 25.5 mg *trans*-rhapontin, 16.0 mg *cis*-rhapontin and 20.5 mg *trans*-desoxyrhaponticin were separated from 80 mg crude sample; the isolates had purities of 99.6, 97.2 and 99.2%, respectively.

Conclusion – A simple and efficient HSCCC method has been optimised for the preparative separation of stilbene glycosides from *Rheum tanguticum* Maxim. ex Balf. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: Preparative separation; *Cis*-rhapontin; *Trans*-desoxyrhaponticin; *Trans*-rhapontin; *Rheum tanguticum* Maxim. ex Balf.

Introduction

Rheum tanguticum Maxim. ex Balf., a perennial herb from the Polygonaceae family, is mainly distributed in the Qinghai, Gansu and northeastern Tibetan areas of China (Guo, 1987). The roots and rhizomes of this plant have been used in traditional Chinese medicinal systems for over 2000 years; its use is recorded in the *ShenNongBenCaoJing*, a famous ancient Chinese medicinal text. The extract of *Rheum tanguticum* Maxim. ex Balf. is known to exhibit various bioactivities, including anti-bacterial, anti-inflammatory, anti-pyretic, anti-oxidant and anti-cancer activities. The extract has also been used as an agent to reduce blood-lipids, blood pressure, obesity and blood urea nitrogen (Zheng and Zhang, 1993). Previous phytochemical studies have demonstrated that anthraquinones, anthracenes and stilbene glycosides were the major bioactive constituents of the plant (Nonaka *et al*., 1977; Jin *et al*., 2006). Among these constituents, stilbene glycosides have recently attracted a great deal of attention due to their pharmacological properties; these properties include anti-HIV, anti-oxidant, anti-tumour, anti-malarial, and anti-allergy activity (Matsuda *et al*., 2001a, 2001b, Matsuda *et al*., 2004; Lv *et al*., 2007; Son *et al*., 2007; Park *et al*., 2008; Lin *et al*., 2010).

Trans-rhapontin (**1**), *cis*-rhapontin (**2**) and *trans*-desoxyrhaponticin (**3**) (Fig. 1 all plant-contained stilbene glycosides (Matsuda et al., 2001a, 2001b; Babu *et al*., 2004), have traditionally been isolated and purified by high-performance liquid chromatography (HPLC) and column chromatography. However, these separation methods are time consuming, require high amounts of organic solvents and typically require numerous steps for completion. For further investigation and development of potential clinical application of these stilbene glycosides, large amounts of the reference compounds at high purity levels are needed. Thus, a more effective and efficient method of isolation and purification of these stilbene glycosides from *R. tanguticum* Maxim. ex Balf. is required.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption in the solid support used in conventional column chromatography and HPLC. It has been widely used in the preparative separation and purification of active components in natural products with similar chemical structures.

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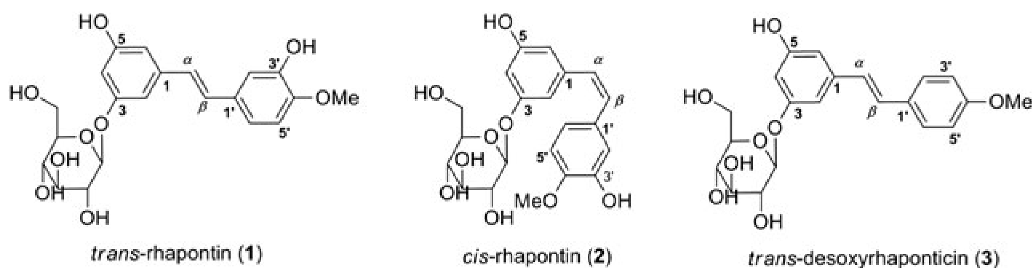

Figure 1. The chemical structures of *trans*-rhapontin (**1**), *cis*-rhapontin (**2**) and *trans*-desoxyrhaponticin (**3**).

To date, few HSCCC separations of stilbene glycosides have been implemented successfully (Cao *et al*., 1998; Jin and Tu, 2005).

To the best of our knowledge, the use of HSCCC for the isolation and purification of *trans*-rhapontin, *cis*-rhapontin and *trans*-desoxyrhaponticin from a herbal medicinal plant has not yet been reported. Previous studies have demonstrated that the roots of *Rhubarb* have a higher content of the stilbene glycosides (Ngoc *et al*., 2008; Püssa *et al*., 2009). The present paper describes a successful preparative separation and purification of three stilbene glycosides from the partially purified extract of the traditional Chinese medicinal plant *Rheum tanguticum* Maxim. ex Balf.

Experimental

Reagents and materials

Roots of *Rheum tanguticum* Maxim. ex Balf. were collected from Gangcha County, Qinghai, China in October 2010, and dried under shade at room temperature. Effective plant root identification was provided by Professor Guichen Chen of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences. *Rheum tanguticum* Maxim. ex Balf. belongs to the genus *Rheum* in the family Polygonaceae, and the voucher specimen (chen201001) was deposited in the Herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai Province.

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from the Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatographic grade, and was purchased from Yuwang Chemical Ltd (Shandong, China). The water used was purified using a PAT-125 (Chengdu ultra Technology Co., Ltd, Chengdu, Sichuan, China) laboratory ultra-pure water system with a 0.4 μm filter.

Apparatus

A TBE-300A high-speed counter-current chromatography instrument (Shanghai Tauto Biotech Co., Ltd, Shanghai, China) with PTFE (polytetrafluoroethylene), three preparative coils (tube diameter: 1.6 mm, total volume: 280 mL) and a 20 mL sample loop was used for HSCCC. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge (*R*), was 5 cm. The β-values of the multilayer coil ranged from 0.5 at 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 revolution speed of the apparatus could be adjusted between a range of 0 and 1000 rpm using a speed controller. The separation temperature was controlled by a HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China). An ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. The system contained a switch valve and a mixer, which were used for gradient formation. The data were collected and analysed with a N2000 workstation (Zhejiang University Star Information Technology Co., Ltd, Hangzhou, Zhejiang, China).

For HPLC, the following equipment was used: an Agilent 1200 system, equipped with a G1354A solvent delivery unit, a G1315B UV–vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an Eclipse XDB-C18 of 5 μm, a 4.6 × 150 mm analytical column, and an Agilent HPLC workstation (Agilent Technologies Co. Ltd, Santa Clara, California, USA). The nuclear magnetic resonance (NMR) spectrometer was a Mercury-400BB NMR (Varian Co. Ltd, Palo Alto, CA, USA) with tetramethylsilane (TMS) as the internal standard.

Preparation of the crude sample

The dried root (200 g) of *R. tanguticum* Maxim. ex Balf. was powdered and extracted three times using 70% ethanol under reflux, each time for 2 h. Then, the residues were concentrated under vacuum. The residues were then suspended in distilled water and extracted with light petroleum (b.p. 60–90 °C, 1.0 L), ethyl acetate (1.0 L), and *n*-butanol (1.5 L), respectively. The *n*-butanol solutions were evaporated to dryness under vacuum at 65 °C to generate 15 g *n*-butanol extract. In order to enrich the targeted components, the extract of *n*-butanol (15 g) was dissolved in deionised water and loaded into a macroporous resin column (90 cm × 4.5 cm, containing 500 g D101 macroporous resin). They were then eluted with various proportions of a mixture of water:ethanol (100:0, 70:30, 30:70 and 10:90 v/v; about 3000 mL for each gradient). The water:ethanol (10:90) fraction was concentrated to produce 2.2 g of crude sample for subsequent HSCCC isolation and purification.

Measurement of the partition coefficient

The partition coefficient was the ratio of the solute distributed between the mutually equilibrated two solvent phases. The partition coefficients were determined by HPLC as follows. A suitable amount of crude sample was added into a series of pre-equilibrated two-phase solvent systems, and the solution was then shaken fully. Subsequently, the same volume of each of upper and lower phases was evaporated to dryness. The residues were diluted into 2 mL methanol and then analysed by HPLC. The *K* value was defined as the peak area of the component in the upper phase divided by the peak area of the component in the lower phase.

Preparation of the two-phase solvent system and sample solution

For HSCCC separation, a two-phase solvent system composed of chloroform:*n*-butanol:methanol:water (4:1:3:2, v/v/v/v) was used. The solvent mixture was thoroughly equilibrated in a separation funnel at room temperature, and the two phases were separated immediately before use. The upper phase was used as the stationary phase and the lower phase was used as the mobile phase. The sample solution for HSCCC separation was prepared by dissolving 80 mg of dried crude powder in 10 mL of the upper phase.

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HSCCC separation procedure

First, the multilayer coil column was entirely filled with the upper phase (stationary phase). Then the apparatus was rotated at 800 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 1.8 mL/min. After hydrodynamic equilibrium was reached, as indicated by the emergence of the mobile phase front, a 10 mL sample solution containing 80 mg of the crude powder was injected into the column through the injection valve by an ÄKTA prime system. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm, and the chromatogram was recorded. The temperature of the apparatus was set to 25 °C. The peak fractions were collected manually according to the elution profile. Evaporation under reduced pressure was then allowed, and the residues were dissolved in methanol for subsequent purity analysis by HPLC. The purity was obtained by HPLC peak area calculation.

HPLC analysis and identification of HSCCC peak fractions

The crude sample and each peak fraction obtained by HSCCC were analysed by HPLC. The HPLC analysis was performed on a reversed-phase Agilent Eclipse XDB-C18 (5 μm, 4.6 × 150 mm) analytical column with gradient elution, at a column temperature of 25 °C. Methanol–water in the following percentages and corresponding times was used as the mobile phase: (methanol: 0–10 min, 25–35%; 10–25 min, 35–40%; 25–40 min, 40–80% and 40–50 min, 80%). The flow-rate and detection wavelength were set at 1.0 mL/min and 280 nm, respectively. The crude sample and peak fractions separated by HSCCC were analysed by HPLC under the optimum analytical conditions, and the chromatograms are presented in Fig. 2. HSCCC peak fraction identification was performed by ^{1}H- and ^{13}C-NMR.

Results and discussion

Selection of solvent system and other HSCCC conditions

Successful HSCCC separation depends upon the selection of a suitable two-phase solvent system. The suitability of the system is dependent on the following conditions (Ito, 1988; Conway, 1990; Oka *et al*., 2002): (i) the settling time of the solvent system should be short (< 30 s); (ii) retention of the stationary phase should be satisfactory; (iii) the partition coefficient (*K*) of the target compound should fall within a suitable range (i.e. usually between 0.2 and 2); the separation factor between the two components ($\alpha = K_1/K_2$, $K_1 > K_2$) should be greater than 1.5 in the semi-preparative multilayer separation column of a commercial HSCCC unit.

In this experiment, several two-phase solvent systems: chloroform–methanol–water, chloroform–*n*-butanol–methanol–water and *n*-hexane–ethyl acetate–methanol–water, were tested according to the polarity of the target compounds, and the results are shown in Table 1. The two-phase solvent systems with *n*-hexane:ethyl acetate:methanol:water (5:5:5:5, 2:5:2:5, 2:5:3:5, 3:11:3:11, 3:12:3:10) were tested first; small *K* values and poor retention of target compounds in the upper phase resulted. Then, two-phase solvent systems comprised of *n*-hexane:ethyl acetate:methanol:water (2:5:3:2, 1:6:1:6, 3:12:3:11) were further investigated. When a ratio of (2:5:3:2, v/v/v/v) was tested, the *K* values were suitable. Nevertheless, the setting time of this solvent system was 32 s (> 30 s), and the retention of the stationary phase was below 30%. When a ratio of (1:6:1:6, v/v/v/v) was used the separation factor between compounds **1** and **2** was too small and not suitable for the separation of the two target compounds from the crude sample. When a ratio of (3:12:3:11, v/v/v/v) was tested the *K* values were between 0.2 and 2, the separation factors between these three compounds were large enough, and the setting time of this solvent system was 26 s (< 30 s). However, the retention of the stationary phase was only 37.9%, and the purity values of the three compounds separated by HSCCC were all below 70%. When a two-phase solvent system comprised of chloroform:methanol:water (4:3:2, v/v/v) was used for the separation, the resulting *K* values were too large, and the test resulted in a long separation time and broad peaks. Fortunately, with the addition of *n*-butanol to

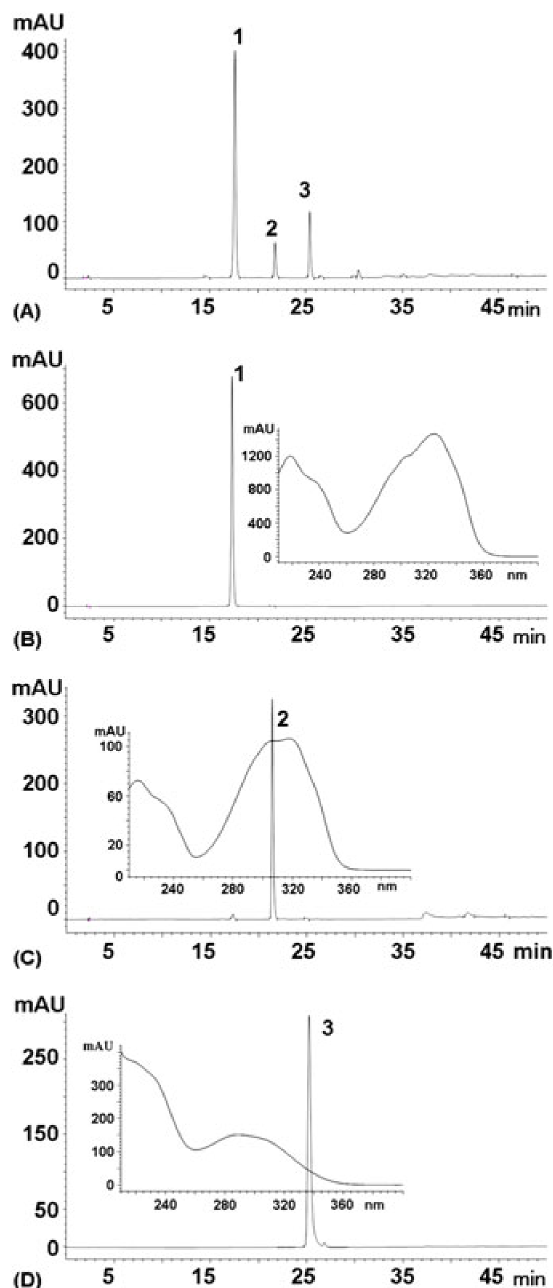

Figure 2. HPLC chromatograms of the crude extract from *Rheum tanguticum* Maxim. ex Balf. (A) After clean up by silica-gel column chromatography and (B–D) the three targeted compounds (peak fractions **1**, **2** and **3**,) purified by HSCCC. Conditions: column: Eclipse XDB-C_{18}-column (250 mm × 4.6 mm i.d., 5 μm); mobile phase: methanol: water (methanol: 0–10 min, 25–35%; 10–25 min, 35–40%; 25–40 min, 40–80%; and 40–50 min, 80%); flow-rate: 1.0 mL/min; column temperature: 25 °C; and detection wavelength: 280 nm.

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Table 1. The *K* values of the target compounds in different solvent systems

Solvent systems	Ratio (v/v)	Settling time (s)	*K* values		
			1	**2**	**3**
n-hexane:ethyl acetate:methanol:water	5:5:5:5	19	0.02	0.12	0.04
n-hexane:ethyl acetate:methanol:water	2:5:2:5	20	0.02	0.10	0.24
n-hexane:ethyl acetate:methanol:water	2:5:3:5	17	0.06	0.11	0.23
n-hexane:ethyl acetate:methanol:water	3:11:3:11	18	0.16	0.22	0.97
n-hexane:ethyl acetate:methanol:water	3:12:3:10	25	0.14	0.23	0.96
n-hexane:ethyl acetate:methanol:water	2:5:3:2	32	0.35	1.87	1.15
n-hexane:ethyl acetate:methanol:water	1:6:1:6	28	0.27	0.29	2.06
n-hexane:ethyl acetate:methanol:water	2:12:3:11	24	0.24	0.38	1.25
chloroform:methanol:water	4:3:2	10	140.45	19.90	9.70
chloroform:*n*-butanol:methanol:water	4:0.2:3:2	12	19.74	2.89	1.45
chloroform:*n*-butanol:methanol:water	4:1:3:2	18	0.95	1.48	0.27

chloroform:methanol:water (4:3:2, v/v/v), the retention of the stationary phase improved. There was also an increase in retention of target compounds in the upper phase. The *K* values of the three target compounds were between 0.2 and 2.0, and the separation factors were large enough for the three stilbene glycosides to exhibit large resolutions relative to one another ($\alpha_{1\ 2} = 1.56\ (K_2/K_1)$, $\alpha_{1\ 3} = 3.52(K_3/K_1)$). The use of chloroform:*n*-butanol:methanol:water (4:1:3:2, v/v/v/v) obtained both appropriate resolution and retention of the stationary phase (about 70%).

In addition to developing a suitable two-phase solvent system, other factors were also investigated: the flow rate of the mobile phase and the revolution speed. Different flow rates of the mobile phase (1.5, 1.8 and 2.2 mL/min) were tested in order to determine their effect on separation time, stationary phase retention and targeted compound purity (Table 2). Results indicated that the low flow rate of the mobile phase led to a lengthy separation time; a low flow rate of the mobile phase could improve the peak resolution and increase stationary phase retention. The increase of the flow rate of the mobile phase resulted in peak resolution decline and a reduction of stationary phase retention; the increase of the flow rate in the mobile phase could speed up the elution of peaks. Under the highest flow rate (2.2 mL/min), the purity of compounds **2** and **3** were only 91.1% and 96.2%, respectively. Considering the elucidated separation time and the purity of the targeted compounds, a flow rate of 1.8 mL/min was used in subsequent HSCCC separation procedures. Additionally, the revolution speed could also impact stationary phase retention, and the high revolution speed is likely to cause emulsification (Han *et al.*, 2010). Thus, we mainly considered the 800 rpm speed in our isolation procedure.

Table 2. Comparison of separation time, stationary phase retention and purities of the three compounds under different flow rates

Flow rate (mL/min)	Separation time (min)	Retention (%)	Purity (%)		
			1	**2**	**3**
1.5	320	70.3	99.7	98.7	99.5
1.8	280	66.9	99.6	97.2	99.2
2.2	260	60.8	98.7	91.1	96.2

HSCCC separation

Under the optimised conditions, in only one round of HSCCC separation and in less than 6 h (the HSCCC chromatogram is shown in Fig. 3, three fractions (**1**, **2** and **3**) were obtained: 25.5 mg *trans*-rhapontin (**1**), 16.0 mg *cis*-rhapontin (**2**) and 20.5 mg *trans*-desoxyrhaponticin (**3**). The HPLC analysis of each of the HSCCC fractions revealed that the purities of these three compounds were 99.6, 97.2 and 99.2%, respectively (Fig. 2). With the exception of *cis*-rhapontin, the compounds obtained could be used as reference substances for chromatographic purposes without additional clean-up.

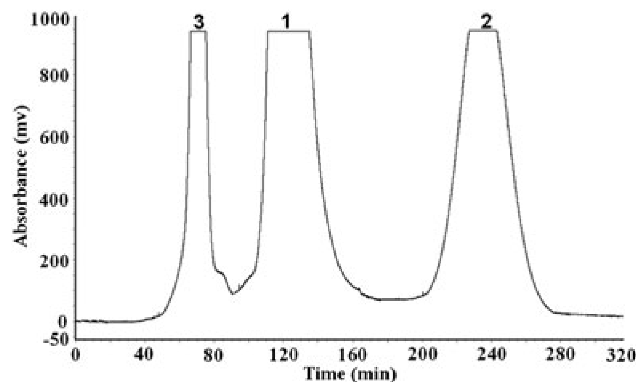

Figure 3. HSCCC chromatogram of the crude extract from *Rheum tanguticum* Maxim. ex Balf. after clean up by macroporous resin column chromatography. Two-phase solvent system: chloroform:*n*-butanol: methanol:water at a ratio of 4:1:3:2 (v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 1.8 mL/min; revolution speed: 800 rpm; detection wavelength: 280 nm; sample size: 80 mg of crude sample dissolved in 10 mL of the upper phase; and separation temperature: 25 °C.

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Identification of the separation peaks

According to ^{1}H-NMR and ^{13}C-NMR data, the chemical structures of the peak fractions separated by HSCCC were identified. Through comparison with reference data, peak **1**, peak **2** and peak **3** were identified effectively as *trans*-rhapontin, *cis*-rhapontin and *trans*-desoxyrhaponticin, respectively. Results for each peak fraction were as follows.

Peak fraction I. Colourless needles, ^{1}H-NMR (DMSO-d_6, 400 MHz): $\delta = 7.02$ (1 H, d, $J = 1.9$ Hz, H-2′); 6.97 (1 H, d, $J = 16.3$ Hz, olefinic H); 6.94 (1 H, dd, $J = 8.4$ Hz, $J = 2.0$ Hz, H-6′); 6.88 (1 H, d, $J = 8.3$ Hz, H-5′); 6.84 (1 H, d, $J = 16.3$ Hz, olefinic H); 6.78 (1 H, s, H-6); 6.61 (1 H, s, H-2); 6.46 (1 H, s, H-4); 4.89 (1 H, d, $J = 7.3$ Hz, sugar-H); 3.93 (1 H, dd, $J = 10.9$ Hz, $J = 2.2$ Hz, sugar-H); 3.85 (3 H, s, OMe); 3.71 (1 H, q, $J = 5.9$ Hz, sugar-H); 3.49–3.45 (3 H, m, sugar-H); and 3.41 (1 H, t, $J = 9.3$ Hz, sugar-H). ^{13}C-NMR (DMSO-d_6, 400 MHz) $\delta = 139.8$ (C-1), 105.7 (C-2), 158.2 (C-3), 102.9 (C-4), 159.1 (C-5), 107.1 (C-6), 126.1 (C-α), 128.6 (C-β), 130.7 (C-l′), 112.3 (C-2′), 146.3 (C-3′), 147.7 (C-4′), 111.3 (C-5′), 118.8 (C-6′), 101.0 (Glc-1), 73.6 (Glc-2), 76.7 (Glc-3), 70.1 (Glc-4), 76.8 (Glc-5), 61.2 (Glc-6) and 55.0 (OMe). The ^{1}H-NMR and ^{13}C NMR data were in agreement with *trans*-rhapontin data found in the literature (Nyemba *et al*., 1995; Aburjai, 2000).

Peak fraction II. Colourless needles, ^{1}H-NMR (DMSO-d_6, 400 MHz): $\delta = 6.80$ (1 H, d, $J = 8.3$ Hz, H-6′); 6.69 (1 H, d, $J = 1.7$ Hz, H-2′); 6.65 (1 H, dd, $J = 8.2$ Hz, $J = 1.7$ Hz, H-5′); 6.39 (1 H, d, $J = 12.8$ Hz, olefinic H); 6.38 (1 H, brs, H-6); 6.32 (1 H, brs, H-2); 6.31 (1 H, d, $J = 11.9$ Hz, olefinic H); 6.28 (1 H, brs, H-4); 4.61 (1 H, d, $J = 7.6$ Hz, sugar-H); 3.73 (3 H, s, OMe); 3.57 (1 H, d, $J = 10.2$ Hz, sugar-H); 3.47–3.44 (1 H, m, sugar-H); and 3.19–3.12 (4 H, m, sugar-H). ^{13}C-NMR (DMSO-d_6, 400 MHz) $\delta = 139.2$ (C-1), 107.8 (C-2), 158.6 (C-3), 103.1 (C-4), 159.0 (C-5), 109.2 (C-6), 128.8 (C-α), 129.9 (C-β), 130.4 (C-l′), 116.2 (C-2′), 146.6 (C-3′), 147.5 (C-4′), 112.4 (C-5′), 120.3 (C-6′), 101.2 (Glc-1), 73.6 (Glc-2), 77.0 (Glc-3), 69.8 (Glc-4), 77.2 (Glc-5), 60.9 (Glc-6) and 56.0 (OMe). The ^{1}H-NMR and ^{13}C-NMR data were in agreement with *cis*-rhapontin data found in the literature (Hui *et al*., 2011).

Peak fraction III. Colourless needles, ^{1}H-NMR (DMSO-d_6, 400 MHz): $\delta = 7.50$ (2 H, d, $J = 8.7$ Hz, H-2′, 6′); 7.06 (1 H, d, $J = 16.4$ Hz, olefinic H); 6.93 (1 H, d, $J = 8.3$ Hz, H-3′, 5′); 6.92 (1 H, d, $J = 16.9$ Hz, olefinic H); 6.73 (1 H, s, H-6); 6.57 (1 H, s, H-2); 6.33 (1 H, s, H-4); 4.79 (1 H, d, $J = 7.7$ Hz, sugar-H); 3.75 (3 H, s, OMe); 3.71 (1 H, d, $J = 10.9$ Hz, sugar-H); 3.47 (1 H, q, $J = 5.8$ Hz, sugar-H); 3.29–3.25 (2 H, m, sugar-H); 3.20 (1 H, t, $J = 8.7$ Hz, sugar-H); and 3.15 (1 H, t, $J = 9.2$ Hz, sugar-H). ^{13}C-NMR (DMSO-d_6, 400 MHz) $\delta = 139.6$ (C-1), 105.3 (C-2), 158.9 (C-3), 103.5 (C-4), 159.4 (C-5), 107.8 (C-6), 126.7 (C-α), 128.6 (C-β), 130.1 (C-l′), 128.3 (C-2′), 114.6 (C-3′), 159.5 (C-4′), 114.6 (C-5′), 128.3 (C-6′), 101.2 (Glc-1), 73.8 (Glc-2), 77.2 (Glc-3), 70.3 (Glc-4), 77.6 (Glc-5), 61.2 (Glc-6) and 55.6 (OMe). The ^{1}H-NMR and ^{13}C-NMR data were in agreement with *trans*-desoxyrhaponticin data found in the literature (Ko, 2000).

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