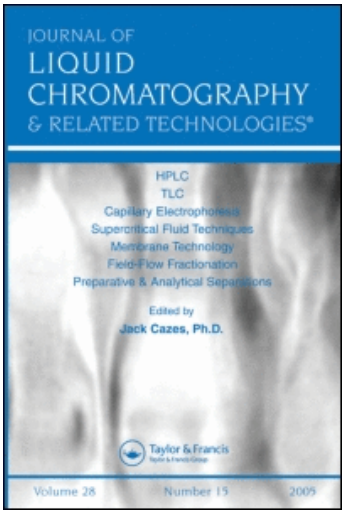

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ISOLATION AND PURIFICATION OF PHENOLIC COMPOUNDS FROM *MAGNOLIAE OFFICINALIS* BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Online publication date: 08 February 2010

To cite this Article Liu, Xia , Tang, Yuhai , Wei, Saili , Yu, Hongling , Lv, Haiyan and Ge, Hongli(2010) 'ISOLATION AND PURIFICATION OF PHENOLIC COMPOUNDS FROM *MAGNOLIAE OFFICINALIS* BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 33: 4, 431 — 440
To link to this Article: DOI: 10.1080/10826070903574063
URL: http://dx.doi.org/10.1080/10826070903574063

Journal of Liquid Chromatography & Related Technologies, 33:431–440, 2010

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903574063

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ISOLATION AND PURIFICATION OF PHENOLIC COMPOUNDS FROM *MAGNOLIAE OFFICINALIS* BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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□ *A rapid, facile, and economical method to isolate and purify phenolic compounds (honokiol, magnolol, and 4-O-methylhonokiol) from the Chinese traditional herb* Magnoliae officinalis *was described. The isolation of honokiol, magnolol, and 4-O-methylhonokiol were performed by preparative high performance liquid chromatography (HPLC) on a* C_{18} *(300 × 50 mm, 10 μm) column using mobile phase methanol and 1% (v/v) acetic acid in water (85/15, v/v), at a flow rate of 85 mL/min and with a detection wavelength of 294 nm. The purified compounds were characterized by* ^{1}H *and* ^{13}C *NMR. The technique described in this article is a fast, economical, and highly efficient tool in the large scale isolation of phenolic compounds.*

Keywords honokiol, HPLC, *Magnoliae officinalis*, magnolol, 4-*O*-methylhonokiol, preparative HPLC, purification

INTRODUCTION

Magnolia officinalis is one widely accepted traditional Chinese medicine, and its root and stem can be used for the treatment of thrombotic stroke, gastrointestinal complaints, anxiety, and so on. The major active constituents of this herb are phenolic compounds, such as honokiol, magnolol and 4-*O*-methylhonokiol (Figure 1). Honokiol and magnolol are isomers, and their difference is only in their positions of one hydroxy group. Honokiol has been found to have stronger pharmacological effects than magnolol. Moreover, honokiol is a small molecule that has been demonstrated to have anti-angiogenic and antitumor properties in diverse tumors, including sarcomas, and chronic lymphocytic leukemia. Recent studies have demonstrated that honokiol overcomes conventional drug resistance

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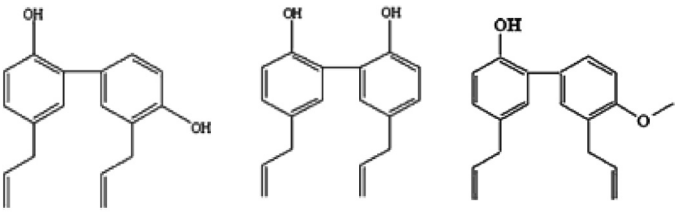

FIGURE 1 Chemical structure of (a) honokiol, (b) magnolol and (c) 4-*O*-methylhonokiol.

in human multiple myeloma by induction of caspase dependent and independent apoptosis.[1] It also efficiently scavenges superoxide radicals in xanthine oxidase and cytochrome P-450 cell free systems, which is similar to the reactivity of ascorbic acid, but above twenty times higher than the reactivity of vitamin E analog trolox. Honokiol potently scavenges intracellular superoxide radicals within melanoma cells.[2]

A novel neolignan compound 4-*O*-methylhonokiol has been isolated from *M. officinalis* and its pharmacologic effects have been investigated in recent years. Kim, Y.H and coworkers,[3–5] had used silica gel column chromatography to isolate 4-*O*-methylhonokiol and it evidently revealed that 4-*O*-methylhonokiol can promote neurite outgrowth through increasing generation of neurotrophic factor[3] and have the anti-inflammatory effect through inhibition of the NF-κB pathway.[4] Also, it may be useful for the prevention of the development and progression of Alzheimer's disease.[5]

In order to perform studies on the biological effects of honokiol, magnolol, and 4-*O*-methylhonokiol in animal or clinical trials, it is necessary to obtain reasonable large quantities of highly pure compounds for experimental purposes. In studies up to now, the separation of the honokiol and magnolol by high speed countercurrent chromatography[6] and preparative high performance liquid chromatography (HPLC)[7] have been reported. This paper describes the extraction method of crude extract from *M. officinalis* by ethanol, and the further purification method that adopted the alkali to dissolve the concentrated ethanol extract according to the major phenolic compounds characterized by weak acid, the addition of acid to release the phenolic compounds. This method could offer three major compounds (honokiol, magonol, and 4-*O*-methylhonokiol) that had been directly separated and purified by preparative HPLC. We describe the development and optimization of preparative RP-HPLC parameters for purification of phenolic compounds through analytical HPLC. At the basis of parameters obtained from analytical HPLC, the optimization of the flow rate and injection volume of the preparative chromatography had been performed. In the end, the highly purified compounds were

characterized by ^{1}H NMR and ^{13}C NMR. The process of extraction, separation, and purification was fast and economical. This method could be used as a guide to scale up the industrially preparative separation of honokiol and magonol.

EXPERIMENTAL

Materials and Reagents

M. officinalis was provided by Huayang Technology Co. Ltd. (Shaanxi, China). Honokiol and magnolol standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products with claimed purities of >98%. Methanol (HPLC-grade) was obtained from Fisher Scientifi (Beijing, China). Other solvents and chemicals used in the present work were of analytical grade.

Preparation of Crude Extract from *M. officinalis*

Dry herbs of *M. officinalis* were ground and sieved to about 60 mesh. Of herb powder, 200 g was loaded into a 2.5 L flask and soaked with 70% (v/v) aqueous ethanol, twice, for 1 h at 90°C in a water bath temperature at the solid liquid ratio of 1:8 (w/v). The extract was dried at 40°C under vacuum in a rotary evaporator. The resulting extract was dissolved by 1.0% NaOH, filtered with a Buchner funnel. The pH of the filtrate was adjusted to 2 by 2.0 M HCl and the precipitate obtained was enriched phenolic compounds, then dried at 40°C in the vacuum drying oven. A crude extract of 200 g was obtained. The crude extract was dissolved in methanol and then filtered through the 0.45 μm membrane for analyzing the weight fractions of phenolic compounds by HPLC.

Analytical Chromatography

Analytical HPLC (Shimadzu, 10Atvp) was performed with a reversed phase C_{18} column (5 μm, 250 × 4.6 mm, Akasil) using LC-10-Atvp double units pumps. The analytical chromatography was carried out under isocratic conditions by varying the percentage of methanol in water at the pH condition of 2.5 adjusted by acetic acid (from 75% to 90%) using a flow rate of 0.8 mL/min at 30°C. Chromatograms were recorded at 294 nm using a SPD-10Atvp variable wavelength detector. Assay samples were dried and dissolved in methanol. A 20 μL sample was injected each time.

Preparative Chromatography Separation

Preparative HPLC separation was carried out on a LabAlliance liquid chromatography system equipped with a Wellchrom K-1800 controller (250 mL pump heads) and a LabAlliance Model 500 dual wavelength absorbance detector. The chromatographic separation was performed on a C_{18} column (300×50 mm, 10 μm), which was obtained from GL Science Inc. (Tokyo, Japan). Detection wavelength was carried out at 294 nm and the products peaks were manually collected.

HPLC Analysis

The collected fractions from preparative HPLC were analyzed using a Shimadzu HPLC apparatus equipped with LC-10-Atvp double units pumps, SPD-10Atvp variable wavelength detector, and a reversed phase C_{18} column (250×4.6 mm, 5 μm, Akasil). The isocratic mobile phase was methanol and 1% (v/v) acetic acid in water (85/15, v/v). The flow rate was 0.8 mL/min at 25°C. Assay samples were dried and dissolved in methanol. Every sample with a 20 μL injection volume was monitored at 294 nm.

^{1}H NMR and ^{13}C NMR

^{1}H NMR and ^{13}C NMR spectra were recorded by Bruker Avence Liquid III 400 NMR (Bruker, Germany) in State Key Laboratory of Applied Organic Chemistry, Lanzhou University. Each spectrum was recorded in $CDCl_3$ using TMS as an internal standard by Bruker Avence Liquid III 400 NMR at 400 MHz.

RESULTS AND DISCUSSION

Purification of Phenolic Compounds and Detection of Their Content in Crude Extract

The solvent extraction was performed according to the literature.[8] According to the major phenolic compounds characterized by weak acid, 1.0% NaOH was used to dissolve the solvent extract after concentration. The pH of alkaline filtrate was adjusted to 2 by 2 M HCl in order to release the phenolic compounds. This method of adding alkaline liquid to purify the pheolic compounds could obtain three major compounds with high contents (Figure 2a). The major separated peaks of a and b, were identified by the standard sample individually. They were honokiol and magnolol, respectively. Peak c was a unknown compound. Averaging the

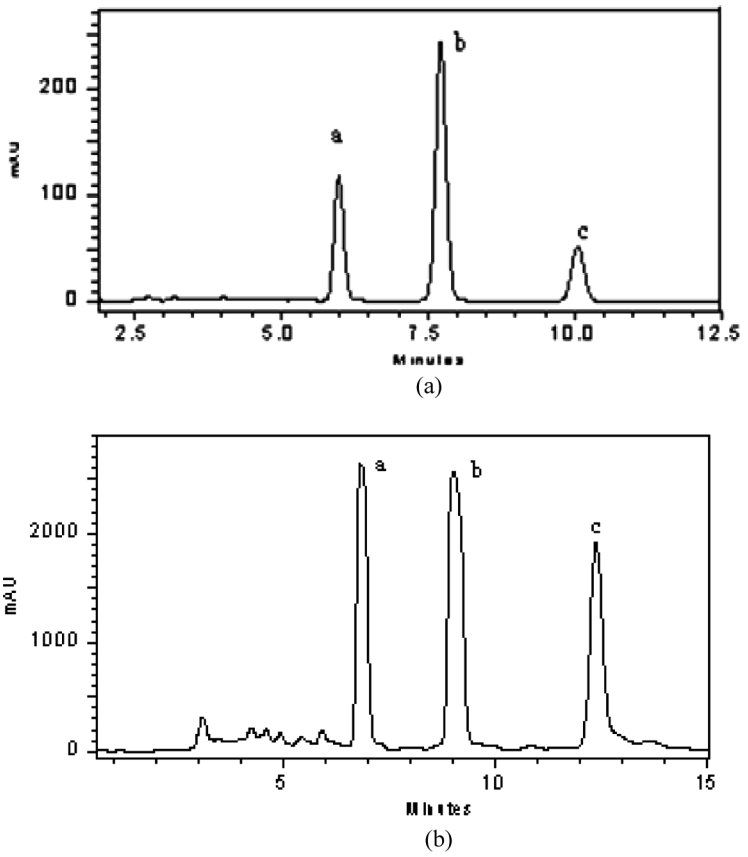

FIGURE 2 The chromatograms of crude extract on the low load of 250 μg/mL (a) and high load product of 10 mg/mL (b). Mobile phase: Methonol-1.0% acetic acid in water (85:15, v/v, pH = 2.5); Flow rate: 0.8 mL/min; Column oven temperature: 30°C; Detected absorbance: 294 nm, Injection volume: 20 μL; Peak identification: a = honokiol, b = magonol, c = unknown.

results of three batches of 2 g of crude extract, the weight fractions of peaks a and peak b were 23% and 52%, respectively. The weight fraction of peak c was 13%, which was determined by an area normalization method. The relative standard deviations (RSD) of the amounts and purities for these repeated extractions were less than 5%.

Selection of the Mobile Phase on the Separation of Phenolic Compounds

The pH of the mobile phase was known to influence the retention of phenolic compounds on the column depending if there is protonation, complete or partial dissociation. Partial dissociation might lead to

additional peak broadening and asymmetric peaks were due to coelution of the acid solute of the component needed to be confirmed and its conjugated base. In all preparative HPLC applications, however, wide injections, which were wider than the peaks at infinite dilution by the mobile phase, were often performed. It easily led to the peak tailing and influenced the resolution between every compound. Adding acid could inhibit the phenolic compounds dissociation, increase their distribution coefficient and retention time, and improve the chromatographic peak profile and resolution. Therefore, 1% acetic acid concentration was used in our study, which was preferable for preparative separation.

The major compounds could be completely separated from the crude extract by the mobile phase, which included methonol-1.0% acetic acid in water (85:15, v/v, pH = 2.5) at 1% acetic acid concentration (pH 2.5) on the low load of 250 μg/mL and the high load product of 10 mg/mL (Figure 2a and b). In the condition of pH 2.5, the effects of the variations in the mobile phase on the retention time, capacity factor (k′), the selectivity factor (α), and the resolution (Rs) of phenolic compounds were investigated in Table 1. The resolution between former adjacent peaks of honokiol and honokiol varied irregularly when the methanol content was changed because of its low quantity. In order to optimize the mobile phase conveniently, the resolutions between honokiol and magnolol, magnolol and unknown compounds had been calculated, respectively.

With the increasing content of methanol in the mobile phase, both the resolutions (Table 1) and the retention times of the solutes went down dramatically. When developing an analytical method for scaling up to the preparative mode, it was desirable to set the value of Rs greater than 2, and that of k′ less than 10. Considering the better separation and the shorter runtime, the application of the mobile phase consisting of methanol-water (85:15, v/v, pH = 2.5) was a reasonable compromise between retention time and resolution.[10]

TABLE 1 Summary of Chromatography Data

Concentration (pH = 2.5)	t_R(min)			k′			α		Rs	
	a	b	c	a	b	c	α_1	α_2	Rs_1	Rs_2
75%	17.2	26.7	43.7	10.5	16.8	28.1	1.60	1.67	11.2	3.7
80%	7.1	9.8	14.1	3.8	5.6	8.4	1.47	1.50	6.6	8.0
85%	6.0	7.7	10.0	3.0	4.2	5.7	1.40	1.36	5.4	6.3
90%	4.8	5.7	7.0	2.2	2.8	3.7	1.27	1.32	3.8	4.9

Note: a is honokiol; b is magonol; c is unknown compound; t_R is retention time(min); k′ is capacity factor; α is separation factor; Rs is resolution; Rs_1 is the resolution of a with b; Rs_2 is the resolution between b and c.

Effect of Flow Rate and Injection Volume on Separation of Phenolic Compounds by Preparative HPLC

In order to decrease the solvent consumption and runtime of the apparatus, the flow rate had to be optimized on the performance of the preparative HPLC separation. The effects of the flow rate upon the separation of phenolic compounds are shown in Table 2. The crude extract of 10 mg/mL concentration was separated by methanol and 1% acetic acid in water (85:15, v/v) as the mobile phase at different flow rates (from 80 to 90 mL/min). Although better amounts, recovery, and purities of products were afforded at the flow rate of 80 mL/min, it had a longer running time on the performing separation by preparative HPLC. Considering the better amount, recovery, purity, and shorter running time together, 85 mL/min was selected as the flow rate of the preparative HPLC.

An overloading study was performed to determine the optimal injection volume (10 mg/mL) of the sample solution. Crude extract solutions of 5, 7, 10 mL without dilution, were injected, respectively. There was no great difference of recovery and purity at the injection volume with 5 mL and 7 mL, respectively (Table 3).

When the injection volume was increased to 10 mL, the recovery and purity of products decreased significantly. In this study, 7 mL of the crude product solution with the concentration of 10 mg/mL was injected per run.

The optimalized flow rate and injection volume were 85 mL/min and 7 mL, respectively. In order to prevent detector saturation, the wavelength

TABLE 2 Effects of Flow Rates Upon the Amount and Purity of Phenolic Compounds (Injection Volume was 5 mL) a is Honokiol; b is Magonol; c is a Unknown Compound

Flow rate (mL/min)	Run time (min)	Amount (mg)			Recovery (%)			Purity (%)		
		a	b	c	a	b	c	a	b	c
80	40	10.0	23.7	6.0	87.0	91.2	92.3	98.56	99.41	94.32
85	28	9.2	22.4	6.2	80.0	86.2	95.4	98.06	99.12	95.04
90	22	8.3	20.5	5.9	72.2	78.8	90.8	94.45	97.17	91.45

TABLE 3 Effects of Injection Volumes Upon the Amount and Purity of Phenolic Compounds (Flow Rate was 85 mL/min) a is Honokiol; b is Magonol; c is a Unknown Compound

Injection volume (mL)	Amount (mg)			Recovery (%)			Purity (%)		
	a	b	c	a	b	c	a	b	c
5	9.2	22.4	6.2	80.0	86.2	95.4	98.06	99.12	95.04
7	14.5	32.7	8.4	90.1	89.8	92.3	98.06	99.12	95.04
10	10.0	35.6	8.3	43.5	68.5	63.8	90.45	92.17	93.45

at 294 nm, instead of the maximum adsorption wavelength, was selected for monitoring. A preparative HPLC chromatogram of crude extract is shown in Figure 3 with methanol and 1% acetic acid in water (85:15, v/v) as the mobile phase at a flow rate of 85 mL/min.

The fraction collection could be exerted according to the retention time or the signal level of the detector.[11] The collection cut points of the fractions were made on the basis of the detector response in our study, so even if a little change of the separation or retention time occurred, the purity of the fractions could be maintained. The horizontal dashed line indicates the starting points of peak a, b, and c, corresponding to the responses of 500, 350, and 200 mAU, respectively (Figure 3). The analytic chromatograms of the collected fractions in view of the detector response are shown in Figure 4. The purities of honokiol and magnolol were both greater than 98%, determined by the peak area normalization method and external standard method. Unknown compound purity determined by area normalization method was greater than 97%.

Structure Determination

The structures of honokiol, magnolol, and 4-*O*-methylhonokiol were characterized by ^{1}H NMR and ^{13}C NMR spectrum as follows: honokiol (Peak a in Figure 3): ^{1}H NMR (400 MHz,$CDCl_3$) δ ppm: 3.334 (2H, d, aryl–CH_2–), 3.442 (2H, d, aryl–CH_2–), 5.036–5.216 (4H, m, 2 × –C=CH_2),

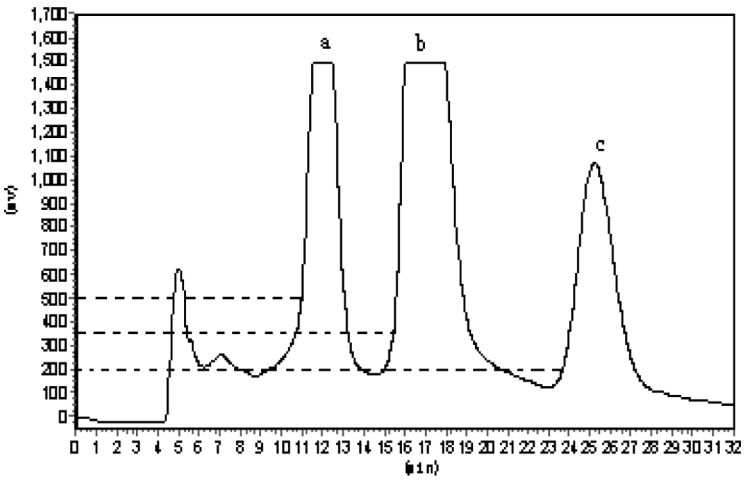

FIGURE 3 Preparative HPLC chromatogram of phenolic compounds. Mobile phase: methanol and 1% acetic acid in water (85:15, v/v). Sample loading: 10 mg/mL crude extract with 7 mL injection volume; Flow rate: 85 mL/min; Detected absorbance: 294 nm. The horizontal dashed line indicates the starting cut points a, b, and c, corresponding to the response at 500, 350, and 200 mAU, respectively.

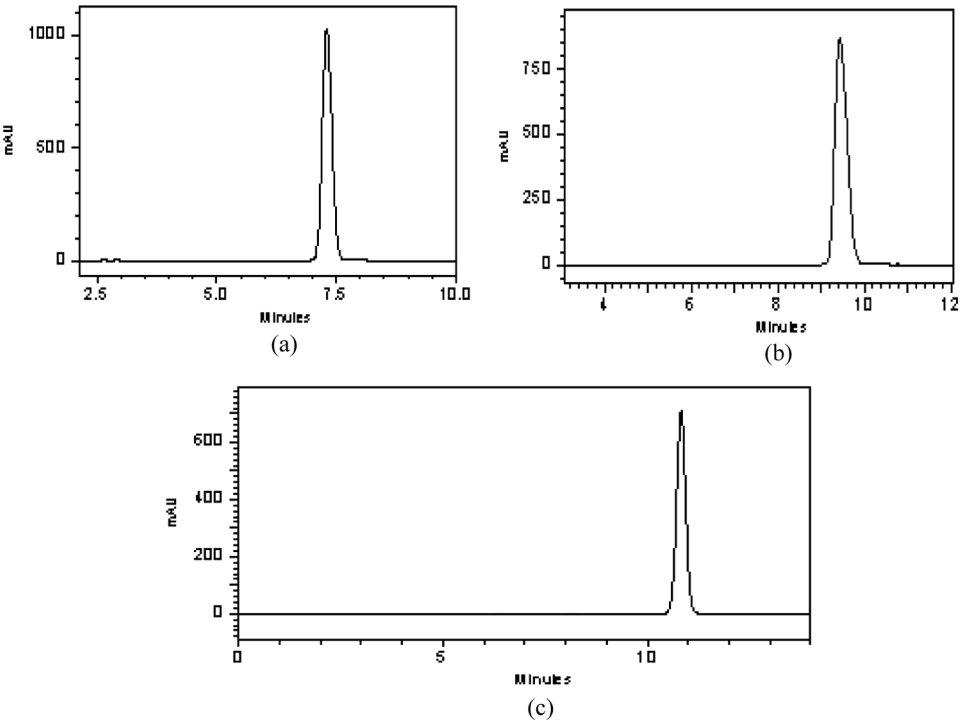

FIGURE 4 HPLC analysis of the fractions corresponding to peak a (a), peak b (b) and peak c (c) obtained from preparative HPLC. Chromatographic conditions as in Fig. 2.

5.360 (2H, brs, 2 × –OH), 5.917–6.082 (2H, m, 2 × –CH=C), 6.884–7.249 (6H, m, ArH). ^{13}C NMR(400 MHz, $CDCl_3$) δ ppm: 116.82 (C-9), 137.77 (C-8), 35.06 (C-7), 131.10 (C-6), 132.21 (C-5), 128.77 (C-4), 115.53 (C-3), 150.74 (C-2), 127.74 (C-1), 115.58 (C-9′), 136.01 (C-8′), 39.37 (C-7′), 128.49 (C-6′), 116.49 (C-5′), 153.90 (C-4′), 129.57 (C-3′), 130.20 (C-2′),126.41 (C-1′). Magnolol (Peak b in Figure 3): ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.372 (2H, d, aryl–CH_2–),3.389 (2H, d, aryl–CH_2–), 5.078–5.130 (4H, m, 2 × –C=CH_2),5.931–6.088 (2H, m, 2 × –CH=C), 6.918 (2H, br s, 2 × –OH),6.940–7.260 (6H, m, ArH). ^{13}C NMR (400 MHz, $CDCl_3$) δ ppm: 39.29 (C-7, 7′), 115.76 (C-9, 9′), 116.67 (C-3, 3′),124.15 (C-1, 1′), 129.79 (C-4, 4′), 131.27 (C-6, 6′),133.23 (C-5, 5′), 137.46 (C-8, 8′), 150.88 (C-2, 2′). According to the literature,[6] peak a was identified as honokiol, peak b was identified as magnolol.

4-*O*-methylhonokiol (Peak c in Figure 3): ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 3.356 (2H, d, aryl–CH_2–), 3.438 (2H, d, aryl–CH_2–), 3.887 (3H, s, −OMe), 5.057–5.205 (4H, m, 2 × –C=CH_2), 5.940–6.074 (2H, m, 2 × –CH=C), 6.905 (1H, d, Ar–H), 6.978 (1H, d, Ar–H), 7.049–7.076(2H, m, ArH), 7.248–7.316(2H, m, ArH). ^{13}C NMR(400 MHz, $CDCl_3$): δ ppm:

115.52 (C-9), 136.49 (C-8), 34.25(C-7), 127.87(C-6), 129.71 (C-5), 157.00(C-4), 128.71(C-3), 130.47 (C-2), 129.06 (C-1), 115.79 (C-9′), 137.78 (C-8′), 39.38 (C-7′), 130.18 (C-6′), 132.11 (C-5′), 115.49 (C-4′), 110.93 (C-3′), 151.82 (C-2′), 127.83 (C-1′), 55.50 (OMe). According to the literature,[5] peak c was 4-*O*-methylhonokiol.

CONCLUSION

A rapid and economical method to isolate and purify phenolic compounds was described in this research. The preparative purification strategy was optimized by a signifcant improvement in the sample overloading volume, flow rate, and cycle time. The purity was assessed by HPLC, ^{1}H NMR, and ^{13}C NMR. A satisfactory result could be achieved with the higher purity and recovery of honokiol, maganol, and 4-*O*-methylhonokiol by using ethanol-water (85:15, v/v, adjusting pH to 2.5 with acetic acid) as a mobile phase on a reversed phase C_{18} column at the 85 mL/min and 294 nm in a fast, economical, and efficient manner.

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