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RAPID COMMUNICATION

HPLC-NNE^{13}CNMR coupling fingerprint analysis technology and its application in a study of *Syringa pubescens* Turcz and its activity against hepatic fibrosis

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Abstract: This study describes the active ingredients of *Syringa pubescens* Turcz which has been identified as being able to protect against hepatic fibrosis. Here we report the characteristics of high performance liquid chromatography and non-nuclear overhauser effect carbon-13 nuclear magnetic resonance (HPLC-NNE^{13}CNMR) technology developed for coupling fingerprint analysis. The major contribution of this new method is the development of an efficient technology and a useful tool for analysis of a traditional Chinese herbal medicine using chromatography and spectral coupling fingerprint technology. Isolation of secoiridoid glycosides and investigation of their structure-activity relationship showed that these derivatives are the active ingredients of *Syringa pubescens* Turcz, and account for the activity of this plant against hepatic fibrosis. The active compounds were identified as oleuropein, 10-hydroxyoleuropein, oleoside-11-methylester, (8Z)-ligstroside, and echinacoside by HPLC-NNE^{13}CNMR coupling fingerprint analysis. A concentration-response relationship was also demonstrated for the HPLC-NNE^{13}CNMR coupling fingerprint method.

Keywords: HPLC-NNE^{13}CNMR, coupling fingerprint, hepatic fibrosis, *Syringa pubescens* Turcz, analysis technology

Introduction

The active ingredients of traditional Chinese medicines are commonly responsible for their pharmacological behavior and therapeutic effects in the clinical setting. Therefore, it is important to understand their mechanisms of action. Traditional Chinese medicines are thought to act on multiple targets via complex mechanisms, so there is no easily identified relationship between their chemical composition and their therapeutic effects. Because phytomedicines play a key role in health care worldwide, much effort has been made internationally to meet requests for product development. The medicinal efficacy of any proposed product should be reasonably potent, the contents of the functional components and substances should be stable and precisely formulated during preparation, and quality control must meet strict manufacturing criteria. The current approaches to quality analysis of a herbal medicine are either compound-oriented or focus on its components. Therefore, fingerprint techniques can be powerful tools in quality control involving traditional Chinese medicines.[1–3] High performance liquid chromatography coupled with non-nuclear overhauser effect carbon-13 nuclear magnetic resonance (HPLC-NNE^{13}CNMR) technology was developed in this study, and represents a newly validated approach for quantitative analysis of herbal medicines.

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Syringa pubescens Turcz is a shrub plant[4] widely distributed in The People's Republic of China,[5,6] growing wild in the Funiu mountain area of Henan province,[7] where the flowers and leaves of the plant have been used for centuries as a folk remedy to treat acute hepatitis, chronic hepatitis, early hepatic cirrhosis, and fatty liver.[4–6] However, to the best of our knowledge, the *S. pubescens* Turcz plant has never been mentioned in the Chinese pharmacopoeia. In the present work, five compounds with activity against hepatic fibrosis were isolated by n-butanol extraction from *S. pubescens* flowers. The individual compounds were structurally identified to have a secoiridoid glycoside skeleton,[8] and the concentration-response relationship was further evaluated by HPLC-NNE[13]CNMR coupling analysis technology.

Materials and methods

Chemicals and apparatus

The silica gel used for thin-layer chromatography and column chromatography was obtained from Qingdao Marine Chem Inc (Shandong Province, People's Republic of China) and the other reagents were of analytical grade and commercially available. All the solvents and samples were filtered through 0.22 μm membrane filters before analysis. Nuclear magnetic resonance spectra were recorded on a DPX-400 spectrometer (Bruker, Ettlingen, Germany). HPLC separation and preparation were performed on an 1100 system (Agilent, Santa Clara, CA, USA) consisting of a binary pump, vacuum degasser, autosampler, column oven, and diode array detector.

Plant materials

Flowers and leaves of *S. pubescens* Turcz were collected from the Funiu mountain area, Songxian, People's Republic of China, in May 2011, and the samples were identified by Changshan Zhu at Henan Agricultural University, Zhengzhou, People's Republic of China. A voucher specimen (20110125) was deposited at the Laboratory of Natural Products, School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology, People's Republic of China.

Sample preparation and compound extraction

A 620 g crude extract was obtained from the fresh flowers and leaves of 10 kg of *S. pubescens* using three 10 L extractions with 70% ethyl alcohol. A previous experiment had confirmed that an n-butanol fraction of 78 g was needed for preparation of a standard extract for therapy in vitro the fibrotic liver models: HSC-T6 hepatic satellite cells, 2.5mg/ml (0.765 ± 0.02, $P < 0.01$).[8] The active ingredient protective against hepatic fibrosis was isolated and purified by column chromatography for HPLC analysis.

Isolation of components from n-butanol fraction of *Syringa pubescens* Turcz with activity against hepatic fibrosis

The chromatographic analysis was performed on a Zorbax SB C_{18} column (250 × 4.6 mm, 5 μm), and the mobile phases for gradient elution consisted of a mixture of acetonitrile and potassium dihydrogen phosphate (20:80). Sample analysis using reverse phase HPLC was run at 1.0 mL per minute and monitored at 254 nm, with oleuropein used as the standard. The injection volume used for the samples was 10 μL. The liquid chromatograms for the oleuropein and n-butanol fractions of *S. pubescens* are shown in Figures 1 and 2, respectively.

Results and discussion

Identification and determination of components with activity against hepatic fibrosis

As shown in Figure 2, five compounds were identified by reverse phase HPLC in the n-butanol extract of *S. pubescens*. These constituents were isolated using column chromatography and their structures were identified by chemical analysis and nuclear magnetic resonance spectra.

In our previous work,[8] these functional components were confirmed to be oleuropein, 10-hydroxyoleuropein, oleoside-11-methyl ester, (8z)-ligstroside, and echinacoside. The chemical structures of these compounds are shown in Figure 3. It is clear that they all have a secoiridoid glycoside skeleton, except for echinacoside (compound 5).

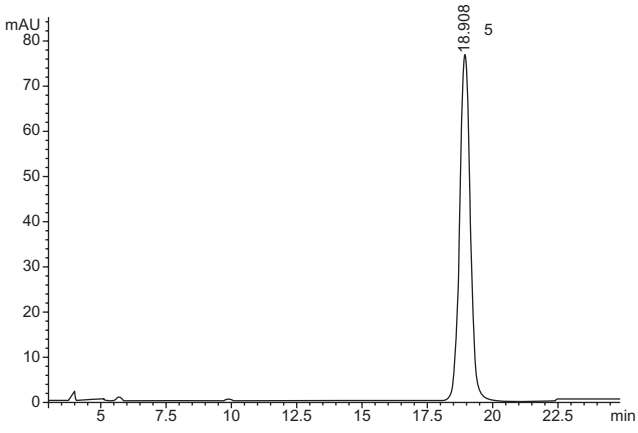

Figure 1 High performance liquid chromatographic analysis of oleuropein.

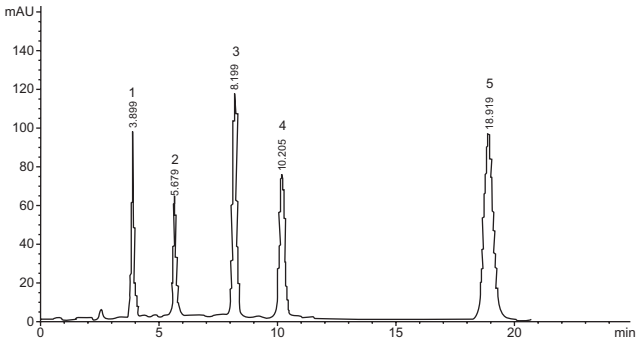

Figure 2 High performance liquid chromatographic analysis of the n-butanol fraction of *Syringa pubescens*.

Further, it was found that the n-butanol extract of *S. pubescens* contained a core ingredient with the ability to inhibit hepatic fibrosis.[9] To demonstrate this activity, there was a need to separate the active substances and to analyze the content of each component in *S. pubescens*. Therefore, HPLC-NNE[13]CNMR coupling fingerprint technology was developed to analyze and evaluate the activity of the components. The NNE[13]CNMR spectra of the secoiridoid glycoside compounds identified in *S. pubescens* is shown in Figure 4. The spectra of these compounds, from bottom to top, represent oleuropein, 10-hydroxyoleuropein, oleoside-11-methyl ester and (8Z)-ligstroside.

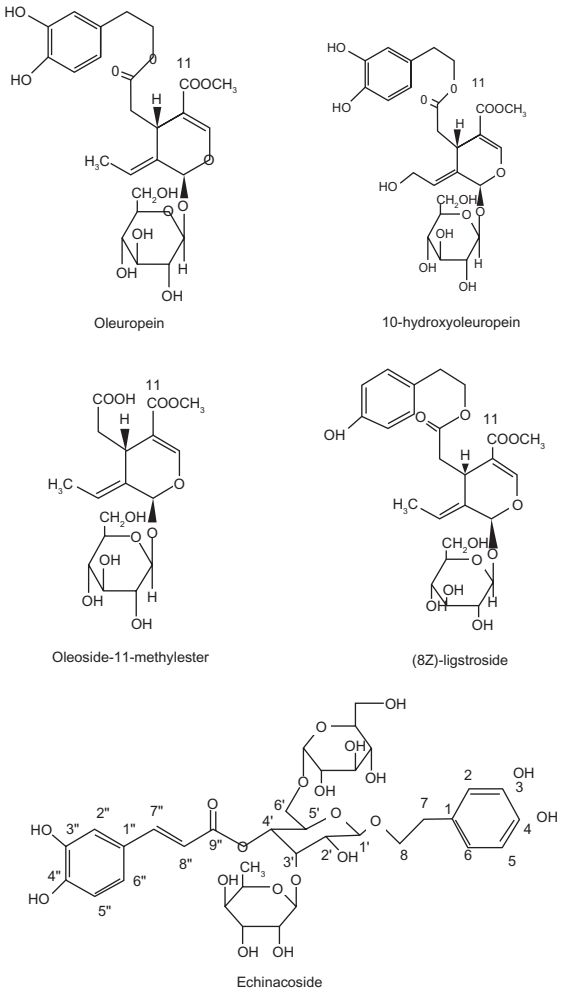

Figure 3 Chemical structure of the five functional components of *Syringia pubescens*.

Based on their structural characteristics, the secoiridoid glycoside derivatives have good solubility in water and in the lipid bilayer. Phytochemical and cytotoxic investigations in various routine bioassays of these natural compounds have shown an important liver-protective effect.[10] It is generally accepted that the secoiridoid compounds have a "catalpa kernel" (iridoid ring structure) as a marker in their molecular structure, and this ring has been reported to have liver-protective activity.[9,10] Our results are in agreement with those reported in literature, ie, the activity of *S. pubescens* flowers and leaves against hepatic fibrosis comes from their secoiridoid glycoside components, as shown in Figure 5, for which fingerprints were provided by HPLC-NNE[13]CNMR coupling technology.

In the present work, a novel HPLC-NNE[13]CNMR coupling method was developed for quantitative analysis of the activity of *S. pubescens* Turcz against hepatic fibrosis and can be used for fingerprinting in the quality control of traditional medicines. More detailed information on the extraction of *S. pubescens* using HPLC analysis showed that the relative peak areas were 95.4% for a total of five compounds; the peak area for the secoiridoid glycosides was 93.0% and that for pure oleuropein was 52.2%. The main components in the n-butanol extract were identified as contributing to the activity of *S. pubescens* against hepatic fibrosis. The minimum effective concentrations for pure oleuropein and n-butanol extract determined by HSC-6 hepatic satellite cell tests were 75 μg/mL and 83 μg/mL, respectively.

Development and characterization of HPLC-NNE[13]CNMR

This method is established and operated as follows: preparation of standard extracts; analysis of standard extracts by HPLC; separation of effective components and structural identification; characterization by NNE[13]CNMR; and establishment of a fingerprint based on HPLC-NNE[13]CNMR coupling technology.

During this process, it is important to understand the analytical procedures used to characterize the reference standard in detail, because they affect both implementation of the method and the data generated for the main compounds identified. For example, when oleuropein was used as the reference standard for assaying the ability of

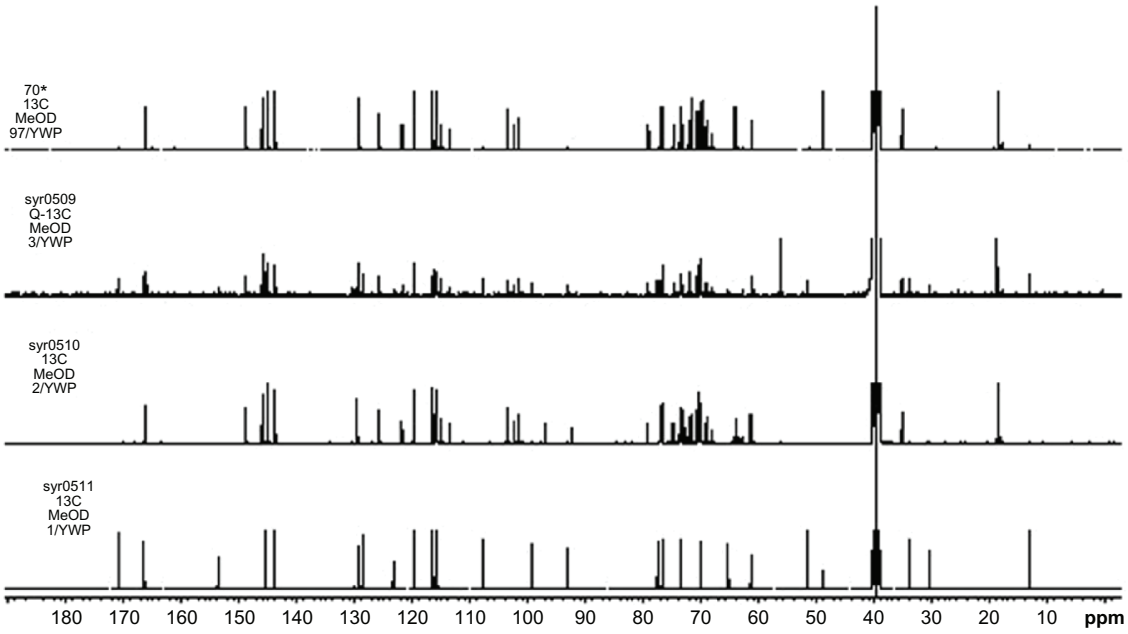

Figure 4 NNE^{13}CNMR spectra for the four secoiridoid glycosides skeleton compounds from *Syringia pubescens*.
Abbreviation: NNE^{13}CNMR, non-nuclear overhauser effect carbon-13 nuclear magnetic resonance.

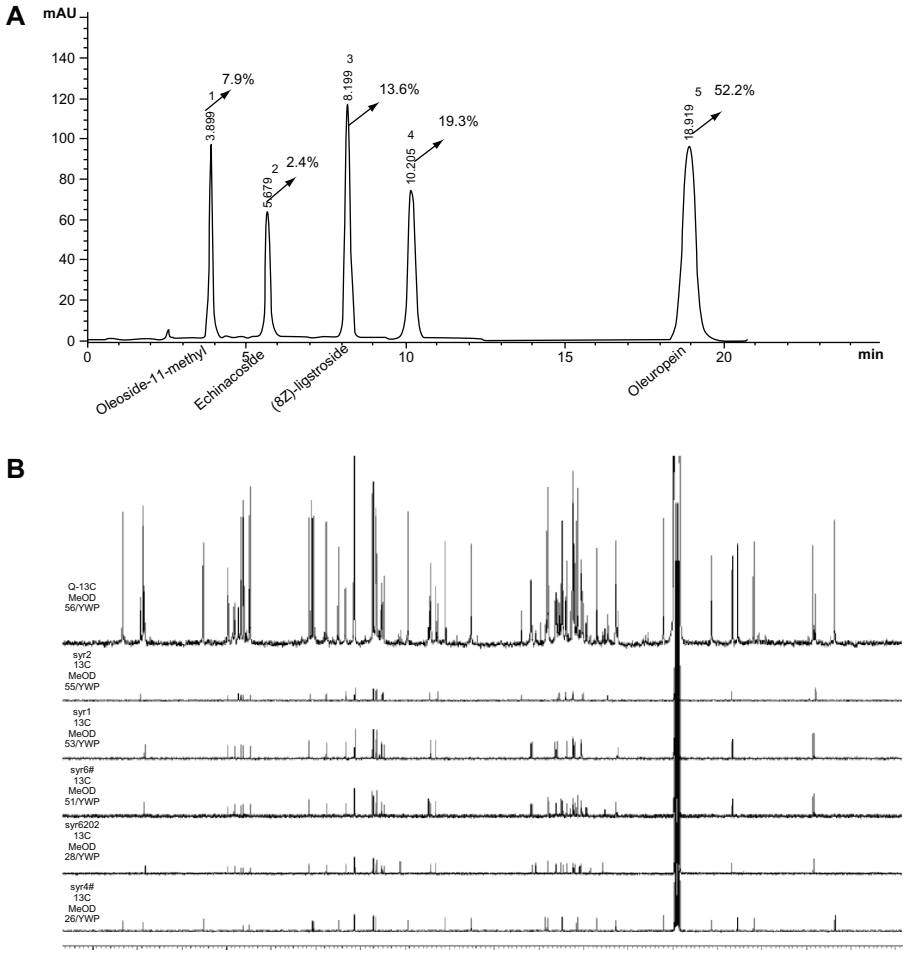

Figure 5 HPLC-NNE^{13}CNMR coupling fingerprints for the components of *Syringia pubescens* with activity against hepatic fibrosis.
Note: (**A**) shows HPLC parts. (**B**) shows one NNE^{13}CNMR parts. A combination of both are HPLC-NNE^{13}CNMR coupling.
Abbreviation: HPLC-NNE^{13}CNMR, high performance liquid chromatography and non-nuclear overhauser effect carbon-13 nuclear magnetic resonance.

the compounds to protect against liver fibrosis, more than 99.5% purity was obtained using the crystal method. These compounds could be identified by thin-layer chromatography and HPLC. As a rule, the reference substance should be the main active component in the extract, and its content should be at least 50%.[11] As noted above, the content of standard oleuropein in this study reached 52.2% in the n-butanol extract of *S. pubescens*. The chemical structures of the five functional compounds from the active ingredients of n-butanol extract of *S. pubescens* are shown in Figure 3. Of note, four of these compounds are secoiridoid glycoside derivatives with hepatoprotective activity. The characteristic signals of NNE[13]CNMR are easily recognized in Figure 4, and partial amplification of the characteristic signal peaks is shown in Figure 5. Structural analysis of the effective constituents can reveal important biological information from HPLC-NNE[13]CNMR fingerprints. Compared with more traditional methods, HPLC-NNE[13]CNMR coupling technology is more advantageous in the qualitative and quantitative control of traditional Chinese medicines. This methodology is experimentally proven to be rational and feasible. In addition, using fundamental principles, fingerprinting of the HPLC-NNE[13]CNMR coupling technology can be deduced and verified using the following formula:

$$\frac{W_1}{M_1}:\frac{W_2}{M_2}=\frac{h_1}{h_2} \quad (1)$$

$$\rightarrow \frac{W_1}{M_1}\frac{M_2}{W_2}=\frac{h_1}{h_2} \quad (2)$$

$$\rightarrow M_1W_2h_1 = W_1M_2h_2 \quad (3)$$

$$\rightarrow W_2 = W_1\frac{M_2}{M_1}\frac{h_2}{h_1} \quad (4)$$

where M_1 is the molecular weight of the reference substance, W_1 is the concentration of the reference substance on HPLC analysis, h_1 is the peak height of the reference substance in NNE[13]CNMR, M_2 is the molecular weight of a compound in the sample, and W_2 and h_2 are the concentration and peak height, respectively, in the NNE[13]CNMR for this compound. The peak that can be easily identified and has no overlap is chosen for the reference substance.

Using the above formula, the actual amount of each component present can be calculated from the HPLC data. For example, if oleuropein is used as the reference substance, measurement of the peak area gives its concentration as 52.2% in the HPLC spectrum, and the relative peak height in the n-butanol extracts provides $h_1 = 4.8$ (δ_C 167.2, C-11, see Table 1 and the NNE[13]CNMR spectrum of syr1 in Figure 5) for oleuropein as well as a peak height of $h_2 = 1.3$ for (8Z)-ligstroside (δ_C 168.6, C-11, see Table 2 and the NNE[13]CNMR spectrum of syr6202 in Figure 5). The content of (8Z)-ligstroside (W_2) can then be calculated using the following formula:

Oleuropein, $W_1 = 52.2\%$, $M_1 = 556.2156$, $h_1 = 4.8$
(8Z)-ligstroside, $W_2 = ?\%$, $M_2 = 539.2129$, $h_2 = 1.3$

$$W_2 = \frac{W_1M_2h_2}{M_1h_1} \times 100\%$$
$$= \frac{0.522 \times 539.2129 \times 1.3}{556.2156 \times 4.8} \times 100\% = 13.64\% \cdot$$

The structure of oleuropein and (8Z)-ligstroside are displayed in Figures 6 and 7. Similarly, the contents of other compounds in standard extracts of *S. pubescens* can be obtained by simple calculation: ie, 10-hydroxyoleuropein 19.3%, oleoside-11-methylester 7.9%, and echinacoside 2.4%. Partial amplification of characteristic signal peaks in the NNE[13]CNMR of the n-butanol extracts is shown in Figure 5. All of the information required can be obtained from these calculations, and the values calculated are consistent with the data obtained by HPLC measurement. Using this method, a valid HPLC-NNE[13]CNMR coupling fingerprint could be created for natural medicines.

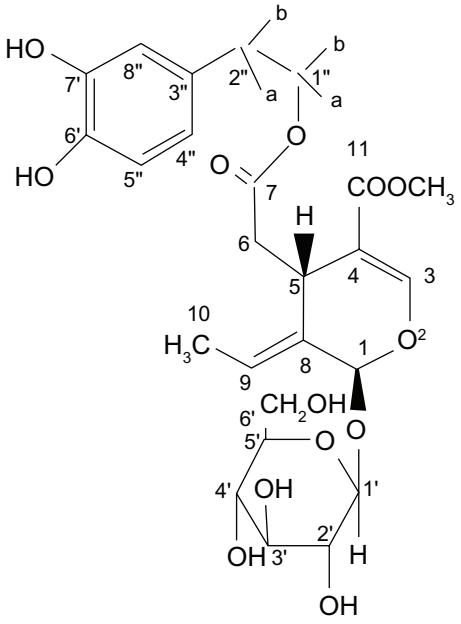

Figure 6 Chemical structure of oleuropein.

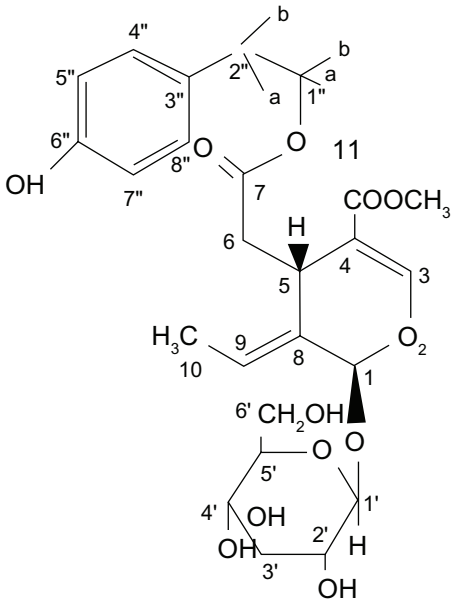

Figure 7 Chemical structure of (8Z)-ligstroside.

Table 1 ^{1}H NMR (MeOD, 400 mHz) and ^{13}C NMR (MeOD,100 mHz) spectral data for oleuropein

Number	δ_H	*J* (Hz)	δ_C
1	5.91		95.1
3	7.50		155.2
4			109.4
5	4.00	9.2, 4.3	31.8
6a	2.44	14, 8.8	41.3
6b	2.70	14, 4.4	
7			173.2
8	6.08	6.8	124.9
9			130.7
10	1.65	7.0	13.6
11			167.2
OMe	3.70		51.9
1′	4.80	8.0	100.9
2′	3.28–3.34		74.7
4′			71.4
5′			78.4
3′	3.79	8.8	77.9
6′a	3.66	12, 5.6	62.7
6′b	3.88	11.6	
1″a	4.09	7.4	66.9
1″b	4.19		
2″	2.75	7.2	35.4
3″			130.5
4″	6.65	2.0	117.0
5″			146.2
6″			144.9
7″	6.68	8.0	116.4
8″	6.54	8.0, 2.0	121.3

Abbreviation: NMR, nuclear magnetic resonance.

Table 2 ^{1}H NMR (MeOD, 400 mHz) and ^{13}C NMR (MeOD, 100 mHz) spectral data for (8Z)-ligstroside

Number	δ_H	δ_C
1	6.04	93.7
3	7.50	154.2
4		112.4
5	3.68	33.8
6a	2.73	37.6
6b	2.86	
7		173.5
8	5.97	126.0
9		132.5
10	1.70	13.5
11		168.6
OMe	3.68	51.8
1′	4.85	100.1
2′		74.8
4′	3.68	70.9
5′		77.2
3′	3.56	77.4
6′a	3.76	
6′b	3.89	62.3
1″	4.13	66.7
2″	2.83	65.4
3″		130.0
4″	7.16	116.2
5″		130.9
6″		157.2
7″	6.57	116.2
8″	7.16	130.9

Abbreviation: NMR, nuclear magnetic resonance.

Conclusion

Because of the complexity of the chemical constituents of traditional Chinese medicines, it is difficult to clarify the structure-activity relationship or to do a concentration-response evaluation by HPLC, gas chromatography, or nuclear magnetic resonance detection alone.[12,13] In the present work, we developed a novel HPLC-NNE^{13}CNMR coupling fingerprint technology that can be used independently to solve some of the existing problems in plant medicine. This method is especially suitable for the same class of skeleton compounds or homologous derivatives. However, the quantitative calculation formula needs to be modified for nonhomologous compounds as follows:

$$W_2 = \varepsilon \frac{W_1 M_2 h_2}{M_1 h_1} \times 100\%$$

where ε is a quantitative relationship model correction factor used to modify the quantitative error between nonhomologous compounds. In short, this novel characteristic HPLC-NNE^{13}CNMR coupling fingerprint is an important tool for

quantitative analysis of multiple ingredients and will be an important area of research in traditional Chinese medicine in the future.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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