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Hypothesis of potential active components in *Angelica sinensis* by using biomembrane extraction and high performance liquid chromatography

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Abstract

The screening and analysis of bioactive components in traditional Chinese medicines (TCMs) is very important not only for the quality control of crude drugs but also for elucidating the therapeutic principle. In this study, a method for screening potential active components from TCMs was developed by using biomembrane extraction and high performance liquid chromatography. Based on the methodology, aqueous extract of *Angelica sinensis* (WEAS) was used, and four compounds were detected by HPLC in the desorption eluate of red cell membrane extraction for WEAS. The compounds were identified as ferulic acid, ligustilide, senkyunolide H and senkyunolide I based on their UV, MS and NMR spectra. Actually, ferulic acid and ligustilide are considered as major active components in *Angelica sinensis*. Therefore, this method may be applied to predict the potential bioactivities of multiple compounds in TCMs simultaneously.

Keywords: Biomembrane extraction; HPLC; *Angelica sinensis*; Hypothesis; Active component

1. Introduction

In most cases, biologically active compounds in traditional Chinese medicines (TCMs) are not or only partially known. Therefore, the screening and analysis of bioactive components in TCMs is very important not only for the quality control of crude drugs but also for elucidating the therapeutic principle. A conventional procedure for finding bioactive components is extraction of TCMs followed by pharmacological screening of the purified compounds. Though some active components such as berberine and artemisinin in Chinese medicine were discovered using this method, the way to increase the probability of success is still controversial, relegating most workers to trial-and-error experiments. In addition, screen of bioactive compounds carried out on animal models are time-consuming, arduous, and inappropriate for directly screening bioactive components from TCMs. Homma et al. [1] introduced the concept of pharmacokinetics, despite the method is effective but laborious. Modern pharmacological studies have shown that combining with some receptors or channels on cell membrane is the first step of drug action. Therefore, the ability of a drug to interact with cell membranes is very important for the behavior of the drug in the organism. In order to study the solute-binding component of cell membranes, a method called retardation chromatography was introduced by Bobinski and Stein [2]. Biochromatography with immobilized protein stationary phases has been applied to probe the interaction between the group of compounds in TCMs and the protein [3–5]. However, the interaction of compounds and human serum albumin is nonspecific. In addition, a single protein is limited for elucidating the curative effect of TCMs, which is an integrative result of a number of bioactive compounds. On the other hand, liposome

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is structurally similar to biological membranes because of its lipid bilayer structure and better fluidity of lipid molecules. Immobilized liposome chromatography was used to study drug–membrane interactions in vitro [6,7]. Indeed, Immobilization of biomembrane or whole cells for chromatography has also attracted much attention in the recent years [8]. However, the cell systems were less stable and the entrapment or immobilization procedure must be adapted to the kind of material that is to be analyzed and to the kind of gel matrix used [9]. In addition, interaction of components in TCMs with biomembrane is rarely compatible to their separation on chromatography. Thus, using biomembrane or whole cells chromatography, the separation and identification of compounds is a problem.

In this paper, a method for screening potential active components in *Angelica sinensis*, one of the commonly used TCMs for tonifying blood, was developed by using human red cell membrane extraction and high performance liquid chromatography, and four potential active candidates in *Angelica sinensis* were hypothesized.

2. Experimental

2.1. Materials

Angelica sinensis was obtained from Minxian County of Gansu Province, China. The identity was confirmed by Professor Qinan Wu, and the voucher specimen was deposited at the National Standard Lab for Chinese Medicine, Nanjing University of Chinese Medicine, Nanjing, China. Ferulic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride were purchased from Sigma (St. Louis, USA). Z-ligustilide was purchased from ChromaDex (Santa Ana, CA, USA). Acetonitrile for LC and acetic acid were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA).

2.2. Extraction of chemical constitutes from Angelica sinensis

About 100 g of ground powder derived from crude herbs was soaked in 800 ml water and then boiled for 1 h. After centrifugation at 3000 rpm for 10 min, the supernatant was collected and lyophilized. Then the extract was dissolved in 100 ml of 5 mM phosphate buffer (pH 7.4) and the fingerprint for water extract of *Angelica sinensis* (WEAS) was determined using HPLC.

2.3. Preparation of human red cell membrane

The membrane was prepared as described by Lundahl et al. [10] with modification. Briefly, volunteer blood was collected into citrated (1:10 dilution, 3.8% sodium citrate) tubes. The blood sample was centrifuged at 800 × *g* for 10 min at 4 °C and the buffy coat and plasma aspirated to remove platelets and white blood cells. Red cells were then washed four times with phosphate buffered saline, and lysed by addition of four-folds of volume 5 mM phosphate buffer (pH 7.4) at 4 °C. After 30 min, the hemolysate was centrifuged at 27,000 × *g* for 90 min and the sedimented material was suspended in 5 mM phosphate buffer to a volume of 50 ml. The membrane was then centrifuged and washed four times with PBS. And the final red cell membrane material was suspended in PBS to a protein concentration of 10 g/l as measured by absorbance at 280 nm [11] and finally the prepared material was used for biomembrane extraction.

2.4. Biomembrane extraction of sample

The same volume of red cell membrane suspension and 5 ml WEAS were incubated with shaking for 30 min at 37 °C. The suspension was then transferred to Centriprep YM-50 centrifugal filters and centrifuged at 1000 × *g* for 10 min or until the filter appears dry. The material collected on the filter was washed three times to remove the unbound components. Each wash was accomplished by addition of 1 ml of PBS to the filter with subsequent centrifugation at 1000 × *g* for 10 min or until dry. The washing eluate were discarded except the last one which was collected as control for HPLC analysis. Finally, red cell membrane was denatured by addition of 1 ml of 20% acetic acid in water to the filter, and the bound components were delivered. The released components were collected by centrifugation into a fresh collection vessel. The desorption eluate from this step is evaporated to dryness by centrifugation in vacuo (SpeedVac, Savant) and reconstituted by sonication in 0.2 ml of a 1:1 acetonitrile/0.25% aqueous acetic acid solution immediately before analysis. The blank desorption eluate, replaced WEAS with phosphate buffer to interact with red cell membrane, was obtained using the method described above, and HPLC analysis of the blank was performed.

2.5. HPLC analysis

Analysis were performed on an Agilent Series 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to an Agilent ChemStation software. A ZORBAX ODS C18 column (4.6 mm × 250 mm i.d., 5 μm) and a ZORBAX ODS C18 guard column (4.6 mm × 12.5 mm i.d., 5 μm) was used. Solvents that constituted the mobile phase were (A) 0.25% aqueous acetic acid and (B) acetonitrile. The elution conditions applied were: 0–10 min, linear gradient 0–2% B; 10–25 min, linear gradient 2–10% B; 25–50 min, linear gradient 10–20% B; 50–65 min, linear gradient 20–35% B; 65–70 min, 35% B isocratic; and finally, reconditioning steps of the column was 0% B isocratic for 15 min. The flow-rate was 1 ml/min and the injection volume was 5 μl. The system operated at 25 °C. Peaks were detected at 280 nm.

2.6. Mass spectrometry

The HPLC conditions for LC–MS analyses were the same as those used for HPLC analysis described on Section 2.5. Agilent 1100 Series LC/MSD Trap (Agilent Technologies, Palo Alto, CA) ion-trap mass spectrometer with electrospray ionization interface, connected to an Agilent ChemStation software was used in HPLC–MS method. ESI-MS conditions were as follows: positive ion mode, drying gas N_2, 10 l/min, temperature 325 °C, pressure of Nebulizer 25 psi, octapole voltage 2.91 V, scan range 50–500 μm. ESI-MS/MS conditions were as follows: positive ion mode, separation width 0.9, fragment amplification 1, scan range 50–400 μm.

2.7. GC–MS analysis

GC–MS was performed with an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). A capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used. The column temperature was at 100 °C for injection, then programmed at 20 °C/min to 280 °C. Split injection (2 μl) was conducted with a split ratio of 1:10 and helium was used as carrier gas of 1.0 ml min^{-1} flow-rate. The spectrometers were operated in electron-impact (EI) mode, the scan range was 50–550 amu, the ionization energy was 70 eV and the scan rate was 0.2 s per scan. The inlet, ionization source temperature were 320 and 300 °C, respectively.

3. Results and discussion

3.1. Biomembrane extraction of compounds in Angelica sinensis

Affinity methods is unique among separation methods as it is the only technique that permits the purification of compounds based on biological functions rather than individual physical or chemical properties [12]. Compounds with affinities similar to a target receptor are also likely to share pharmacological properties. Thus, the affinity characteristics for a series of related molecules provide a delineation of the pharmacophore that is responsible for their biological activity. Several ingenious screening methods have been reported which using affinity chromatography [13–15]. However, the cell systems are less stable and organic solvents can destroy the structure of membrane. The affinity interaction between components in TCMs and biomembrane is rarely compatible to their separation on chromatography. Therefore, it is an optimal method for screening of potential active components in TCMs using biomembrane extraction and HPLC analysis. In addition, mass spectrometry provides the capability to directly identify bound species in the affinity capture.

In this paper, red cell membrane was chosen mainly based on its easy preparation and the therapeutic effects of *Angelica sinensis*. Actually, some other biomembrane could be used to avoid the possibility of potential active compounds might not bind to human red cell membranes. Primary test showed that the fingerprints of the aqueous extract of *Angelica sinensis* detected at 254, 280 and 320 nm were similar (data not shown). However, the number of peaks was more and the signal was more sensitive at 280 nm. Therefore, the components were monitored at 280 nm for HPLC analysis. HPLC Chromatograms of WEAS and desorption eluate of biomembrane interacted with WEAS under the experimental conditions described above were shown in Fig. 1. There are four principal peaks, C1, C2, C3 and C4 when detected at 280 nm. And there is no peak found in the chromatograms of control eluate and blank desorption eluate.

3.2. Identification of detected components

MS is a powerful technique for identification of molecular structure. However, the fractions of peaks separated by immobilized biomembrane affinity chromatography could not be directly applied for MS analysis because of the presence of a high concentration of inorganic salts [7]. Using biomembrane extraction and then HPLC analysis, the components of peaks could be directly identified by MS. The molecular masses of components for peaks C1, C2, C3 and C4 were determined as 194, 224, 224 and 190, respectively. The UV spectra components for peaks C1 and C4 match with ferulic acid and ligustilide very well. Their MS spectra as shown in Fig. 2 also coincided with those of standards. So the compo-

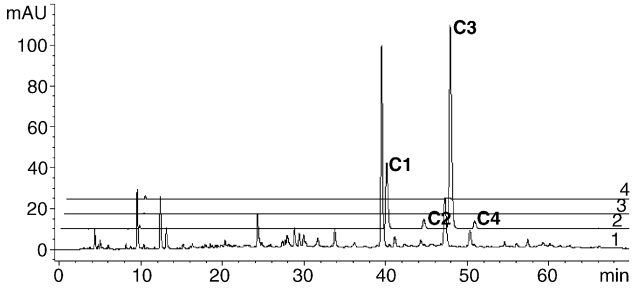

Fig. 1. HPLC chromatograms of aqueous extract of *Angelica sinensis* (WEAS) (1), desorption eluate of biomembrane interacted with WEAS (2), final washing eluate of biomembrane interacted with WEAS (control) (3) and desorption eluate of biomembrane interacted with phosphate buffer (blank) (4) analysis were performed on an Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to an Agilent ChemStation software. A ZORBAX ODS C18 column (4.6 mm × 250 mm i.d., 5 μm) and a ZORBAX ODS C18 guard column (4.6 mm × 12.5 mm i.d., 5 μm) was used. Solvents that constituted the mobile phase were (A) 0.25% aqueous acetic acid and (B) acetonitrile. The elution conditions applied were: 0–10 min, linear gradient 0–2% B; 10–25 min, linear gradient 2–10% B; 25–50 min, linear gradient 10–20% B; 50–65 min, linear gradient 20–35% B; 65–70 min, 35% B isocratic; and finally, reconditioning steps of the column was 0% B isocratic for 15 min. The flow-rate was 1 ml/min and the injection volume was 5 μl. The system operated at 25 °C. Peaks were detected at 280 nm.

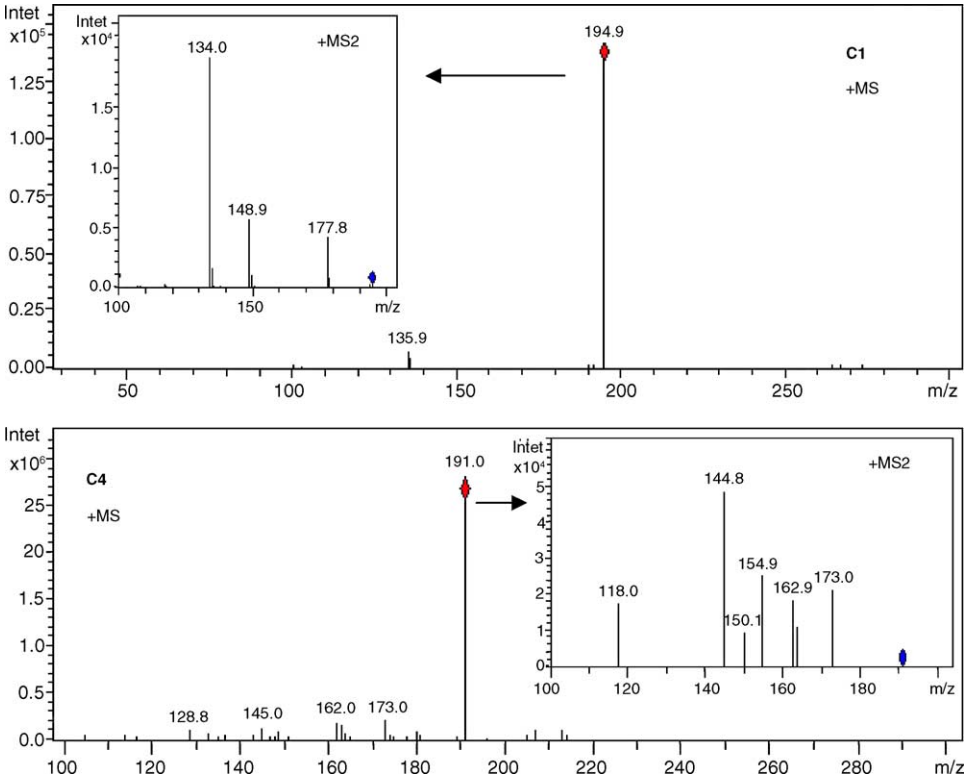

Fig. 2. MS and MS/MS spectra of peaks C1 and C4 in desorption eluate of biomembrane extraction of aqueous extract of *Angelica sinensis* on HPLC. HPLC condition was the same as that described in Fig. 1.

nents for peaks C1 and C4 can be identified as ferulic acid and ligustilide, respectively. Their structures are shown in Fig. 3.

The components for peaks C2 and C3 could not be identified based on their diode array and MS detection coupled with HPLC. Therefore, they were separated and purified by RP-HPLC according to their chromatograms. Then the MS spectra of components for peaks C2 and C3 (0.1 mg/ml in methanol) were detected by GC–MS and shown

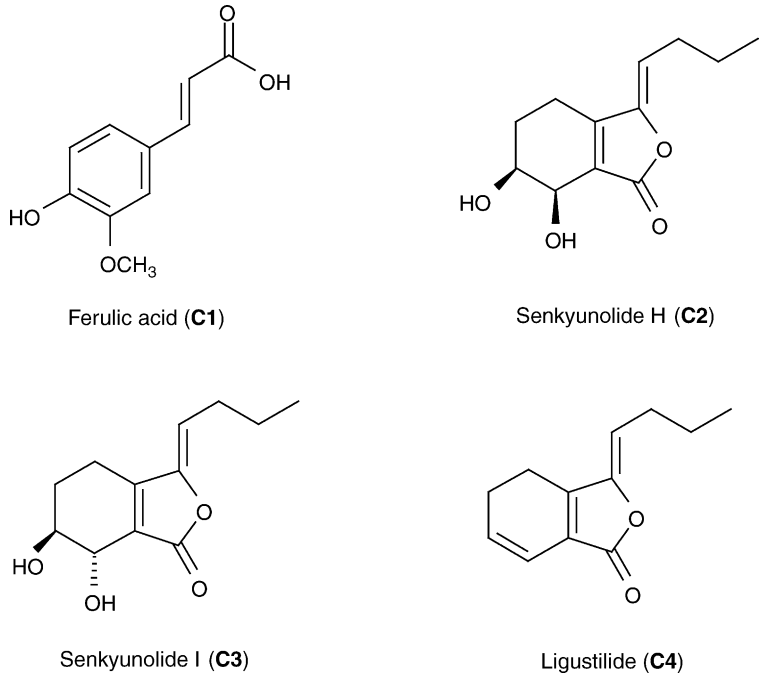

Fig. 3. Structures of ferulic acid (C1), senkyunolide H (C2), senkyunolide I (C3), and ligustilide (C4).

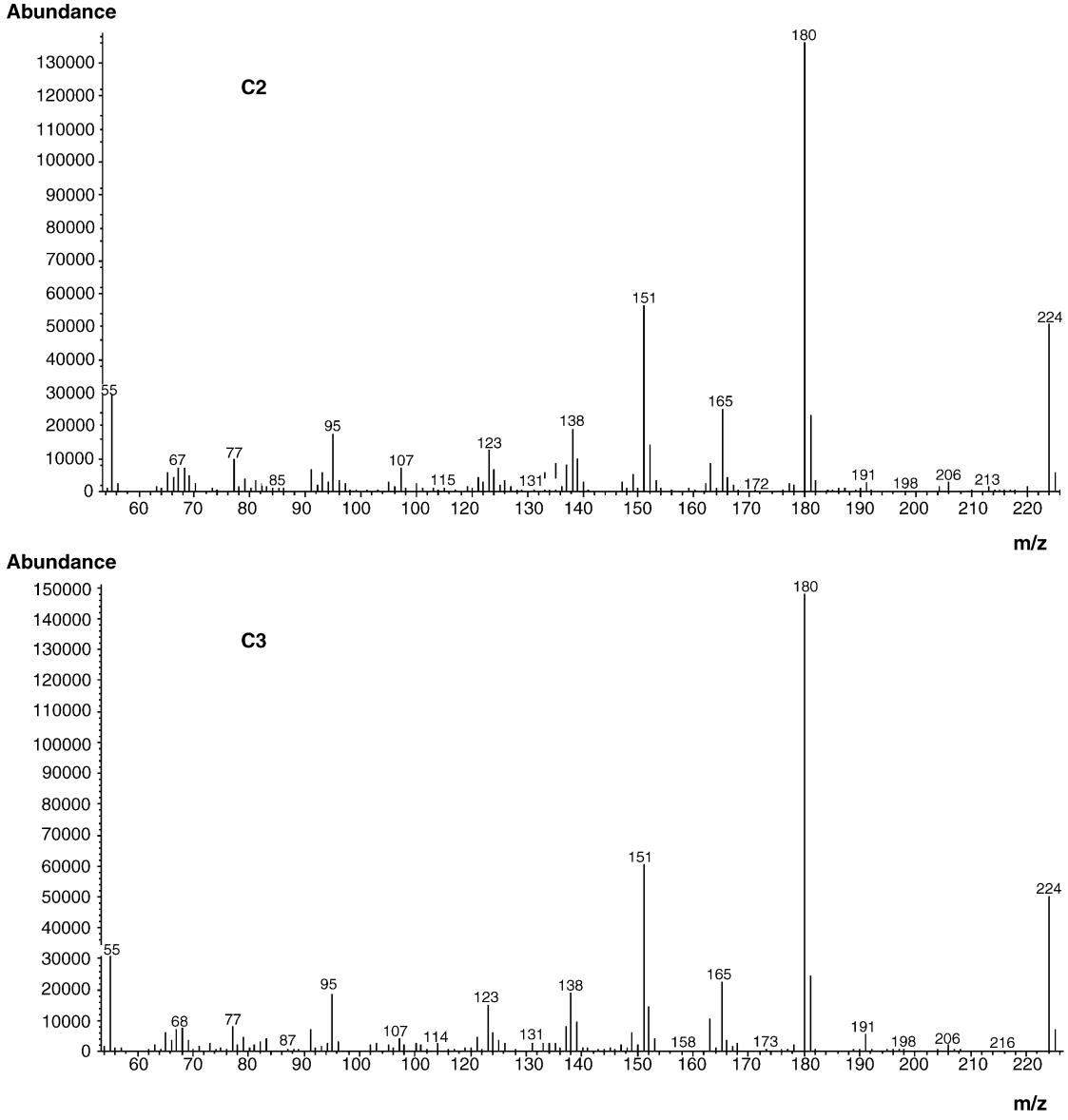

Fig. 4. MS spectra of components for peaks C2 and C3 prepared from aqueous extract of *Angelica sinensis* by HPLC. MS spectra was determined by an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent ChemStation software. A capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used. The column temperature was at 100 °C for injection, then programmed at 20 °C min^{-1} to 280 °C. Split injection (2 μl, 0.1 mg/ml) was conducted with a split ratio of 1:10 and helium was used as carrier gas of 1.0 ml min^{-1} flow-rate. The spectrometers were operated in electron-impact (EI) mode, the scan range was 50–550 amu, the ionization energy was 70 eV and the scan rate was 0.2 s per scan. The inlet, ionization source temperature were 320 and 300 °C, respectively.

in Fig. 4. It can be seen that the components for C2 and C3 have very similar behavior, so it is very possible that the former are a structural analog of the latter. By comparing with literature data [16–18], it can be observed that the mass spectra of peaks C2 and C3 were similar to the mass spectrum of 6,7-dihydroxyligustilide. Senkyunolide-H and senkyunolide-I are an isomeric pair of dihydroxyphthalides, and senkyunolide-I is the major isomer [19]. Based on these facts, peaks C2 and C3 were identified as senkyunolide-H and senkyunolide-I (Fig. 3), respectively. It was further supported by their ^{1}H NMR and ^{13}C NMR data (Table 1).

Angelica sinensis is used for tonifying blood and treating female irregular menstruation and amenorrhoea. It is also used for treatment of anemia, hypertension and cardiovascular diseases. Over 70 compounds, such as, phthalides, terpenes, aromatic compounds, have been isolated and identified from it. Its main essential components, ligustilide, other phthalides and ferulic acid are thought to be the biologically active components [20]. In this study, senkyunolide H and senkyunolide I were also detected as bioactive candidates using biomembrane extraction besides ferulic acid and ligustilide. The pharmacological activities of senkyunolide H and senkyunolide I will be investigated in future.

Table 1
NMR data of the compounds prepared by HPLC for peaks C2 (senkyunolide H) and C3 (senkyunolide I)

Position	^{1}H NMR (δ ppm)		^{13}C NMR (δ ppm)	
	Peak C2	Peak C3	Peak C2	Peak C3
1			169.3	169.2
3			153.2	153.2
3a			148.2	148.1
4	2.40–2.70 (m)	2.45–2.60 (m)	18.4	18.8
5	1.80–2.20 (m)	1.85–2.10 (m)	25.7	26.2
6	4.05 (ddd, J = 7.9, 3.6, 2.6 Hz)	3.95 (ddd, J = 9.3, 5.2, 2.8 Hz)	67.3	71.5
7	4.61 (d, J = 3.6 Hz)	4.46 (d, J = 5.2 Hz)	63.4	67.2
7a			125.4	125.9
8	5.31 (t, J = 7.9 Hz)	5.26 (t, J = 7.9 Hz)	114.3	114.1
9	2.36 (dt, J = 7.9, 7.3 Hz)	2.31 (dt, J = 7.9, 7.4 Hz)	28.1	28.1
10	1.50 (tq, J = 7.3, 7.3 Hz)	1.46 (tq, J = 7.4, 7.3 Hz)	22.3	22.2
11	0.95 (t, J = 7.3 Hz)	0.92 (t, J = 7.3 Hz)	13.7	13.7

NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian Gemini-300 spectrometer (Varian Inc., Palo Alto, CA). TMS was used as internal standard for ^{1}H spectra. $CDCl_3$ was used as solvent.

4. Conclusion

In this study, we present a method for screening and determining potential active components in *Angelica sinensis* by using biomembrane extraction and HPLC. Using biomembrane which has a strong interaction with active compounds, such compounds can be enriched from extract of TCMs. Because the curative effect of TCMs is an integrative result of a number of bioactive compounds, biomembrane extraction may be applied to predict the potential bioactivities of multiple compounds in TCMs simultaneously. In addition, HPLC coupled with other complementary techniques, such as diode array detection, MS, and NMR will further improve the resolution and structural identification of potential active compounds in TCMs.

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