Carbohydrate Polymers 114 (2014) 115–122

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Inhibitory effect of *Angelica sinensis* extract in the presence of 2-hydroxypropyl-β-cyclodextrin

Chin-Mu Hsu[a,b,c], Fuu-Jen Tsai[c,d,e], Yuhsin Tsai[a,c,*]

[a] *Graduate Institute of Chinese Medicine, China Medical University, Taichung, Taiwan*
[b] *Department of Medical Research, China Medical University Hospital, Taichung, Taiwan*
[c] *School of Chinese Medicine, China Medical University, Taichung, Taiwan*
[d] *Department of Medical Genetics, Pediatrics and Medical Research, China Medical University Hospital, Taichung, Taiwan*
[e] *Department of Biotechnology, Asia University, Taichung, Taiwan*

ARTICLE INFO

Article history:
Received 26 January 2014
Received in revised form 15 July 2014
Accepted 19 July 2014
Available online 27 July 2014

Keywords:
Angelica sinensis
2-Hydroxypropyl-β-cyclodextrin
Traditional Chinese medicine
Complex
Hepatoma cell

ABSTRACT

Angelica sinensis (AS) is a traditional Chinese medicinal herb. Its ethanolic extract contains active ingredients, such as ferulic acid, ligustilide, and butylidenephthalide, which are hydrophobic and have inhibitory effects on hepatoma cells. To increase the aqueous solubility/dispersibility of AS extract and study the consequent inhibitory effect on hepatoma cells, the ethanolic extract of AS was complexed with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), a cyclic oligosaccharide that has a hydrophilic outer surface and a hydrophobic central cavity. The AS–HP-β-CD complex (weight ratio of AS extract: HP-β-CD = 1:5) was prepared and characterized. The effect of complexing the AS extract with HP-β-CD on Hep3B cell growth was investigated by analyzing cytotoxicity. Our results showed that cytotoxicity inhibition of AS–HP-β-CD complex was up to 94% and higher than that of AS extract (about 68%). These observations suggested that the use of HP-β-CD to stabilize AS extract in aqueous solution was possible for herbal medicine application.

1. Introduction

Angelica sinensis (Oliv.) Diels (AS), a member of the Umbelliferae family, is one of the most popular traditional medicines in China. The AS rhizome contains more than 80 compounds, most of which have been isolated and identified (Wei et al., 2008; Yi, Liang, Wu, & Yuan, 2009). Among these, ferulic acid, ligustilide, and butylidenephthalide are considered the active ingredients of the AS root (Fig. 1(a)) (Chao & Lin, 2011). Asian people frequently use AS as a daily dietary supplement because of its health-promoting anti-inflammatory-, anti-cancer-, anti-oxidative-, and anti-hepatotoxic effects, coupled with a unique pleasant flavor (Chao & Lin, 2011).

Worldwide hepatocellular carcinoma is one of the cancers with highest mortality and is the most severe complication of chronic liver disease (Parkin, Bray, Ferlay, & Pisani, 2005). Recently, several studies have shown that AS extract inhibits human hepatocellular carcinoma cell growth (Chao & Lin, 2011) by inducing Nur77 orphan nuclear receptor expression (Chen et al., 2008) or enhancing methylguanine methyltransferase (MGMT) promoter methylation (Yu et al., 2010).

Naturally occurring cyclodextrins (CDs) are cyclic oligosaccharides that are composed of (α-1,4)-linked-glucopyranose units. CDs have a hydrophilic outer surface and an inner hydrophobic cavity (Brewster & Loftsson, 2007). The α-, β-, and γ-CDs are most common and consist of 6, 7, and 8 glucopyranose units, respectively. The most beneficial characteristic of these CDs is their ability to act as host molecules in the formation of inclusion complexes in aqueous solutions. Inclusion complexes of native and modified CDs with many different molecules have been widely used in the supramolecular chemistry, pharmacology, food science, and cosmetics fields (Vyas, Saraf, & Saraf, 2008).

Recently, instead of using a single compound as the guest molecule, crude extracts of cinnamon (Ayala-Zavala et al., 2008), garlic (Ayala-Zavala et al., 2008) and olive (Mourtzinos, Salta, Yannakopoulou, Chiou, & Karathanos, 2007) or medicinal herbs (Feng et al., 2008; Hsu, Yu, Tsai, & Tsai, 2013; Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, & Makris, 2010; Michali, Karathanos, Chiou, Karathanos, & Mourtzinos, 2008) were combined with CDs to form inclusion complexes with improved thermal stability (Andreu-Sevilla, Carbonell-Barrachina,

* Corresponding author at: School of Chinese Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung, Taiwan. Tel.: +886 422053366x1019; fax: +886 422032295.
E-mail addresses: yhtsai@mail.cmu.edu.tw, ytsai5168@yahoo.com.tw (Y. Tsai).

http://dx.doi.org/10.1016/j.carbpol.2014.07.042
0144-8617/

Lopez-Nicolas, & Garcia-Carmona, 2011; Michali et al., 2008; Mourtzinos et al., 2007), antioxidant capacity (Kalogeropoulos et al., 2010), and antimicrobial (Ayala-Zavala et al., 2008) and anti-inflammatory (Feng et al., 2008) activities.

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD; Fig. 1(b)), is a hydroxyalkylated CD derivative and is widely used to increase the solubility, stability, and bioavailability of drugs, because it is more water soluble and less toxic than β-CD (Gould & Scott, 2005). Ferulic acid, ligustilide, and butylidenephthalide, the active ingredients of AS, are insoluble in water (Chao & Lin, 2011), which limits its bioavailability. Therefore, the use of HP-β-CD to complex with ethanolic extract of AS to solve the limitations of AS extract and studies of its inhibitory effect on human hepatoma cells are of interest.

In this study, we prepared a complex (AS–HP-β-CD complex) of AS ethanolic extract and HP-β-CD using a freeze-drying method. The active ingredients of AS ethanolic extract and AS–HP-β-CD complex were determined by liquid chromatography–mass spectrometry (LC–MS). Thin layer chromatography (TLC), proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and two-dimensional rotating frame spectroscopy (2D ROSEY) were used to confirm the complex formation. We determined the effects of complexing the AS extract with HP-β-CD on Hep3B hepatoma cell growth by analyzing cytotoxicity and cellular uptake.

2. Experimental

2.1. Materials

HP-β-CD (*MW* ~ 1460 with 0.8 molar substitution, hydroxypropyl moiety) and phosphate buffered saline (PBS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Analytical grade ethanol and TLC plate (DC-Alufolien, Silicagel 60 F254, 0.2-mm thickness) were purchased from Merck (Darmstadt, Germany). Ferulic acid and ligustilide were purchased from Alfa Aesar (Ward Hill, MA) and ChromaDex (Santa Ana, CA), respectively. Acetonitrile (ACN) and formic acid were purchased from JT Baker (Phillipsburg, NJ). Deionized water (18 MΩ cm) from a Milli-Q system (Millipore, Bedford, MA) was used for all experiments. All other reagents were of analytical grade. The (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTS) cell proliferation assay kit was obtained from Promega (Wisconsin, USA).

2.2. Preparation of AS crude extract

The air-dried slices of AS, purchased from a Chinese medicine store (Taichung, Taiwan), were authenticated by pharmacognosists at China Medical University Hospital and were stored at 4 °C. In detail, 100 g of AS were ground, extracted with 500 mL 95% ethanol, and mixed for 24 h at 25 °C using a magnetic stirrer (100 rpm). The infusion was filtered to remove insoluble materials and freeze-dried at −50 °C. The average yield of AS extract was approximately 6% (w:w). The dried extract was stored at −20 °C and was used in all experiments.

2.3. Preparation of AS–HP-β-CD complex

AS ethanolic extract and HP-β-CD were mixed at different weight ratios (AS extract: HP-β-CD = 1:1, 1:3, and 1:5). The solubility test showed the complex with a weight ratio of AS extract: HP-β-CD = 1: 5 had the best solubility. Therefore, this ratio of AS extract: HP-β-CD was used for further experiments. The AS–HP-β-CD complex with different weight ratios was prepared as follows. Different amounts of HP-β-CD (5 g, 3 g, or 1 g) were dissolved in 60 mL 95% ethanol. AS extract (1 g) dissolved in 15 mL 95% ethanol was slowly added to the HP-β-CD solution and stirred continuously for 24 h at 25 °C. The mixture was then froze at −50 °C and lyophilized to produce the AS–HP-β-CD complex, which was used directly for other experiments without further washing process.

2.4. LC–MS

HPLC separation was carried out on an Atlantis T3 C_{18} 5 m 2.1 × 150 mm analytical column (Waters, Millford, MA). The two

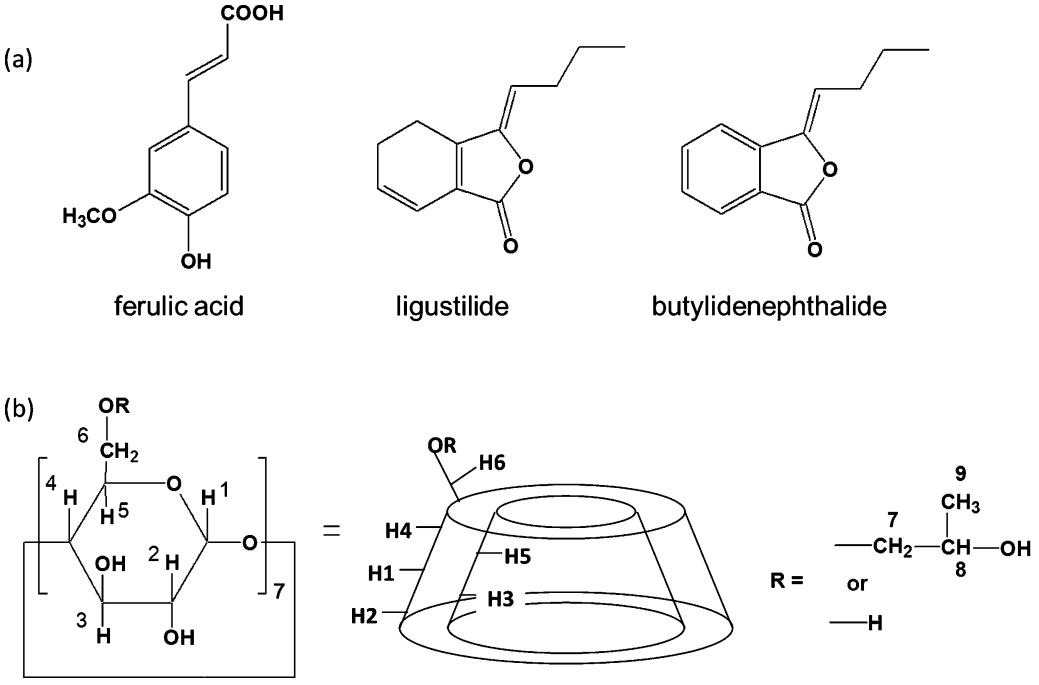

Fig. 1. Chemical structures of (a) ferulic acid, ligustilide, and butylidenephthalide, and (b) HP-β-CD.

mobile phases were buffer A, water (5% ACN and 0.1% formic acid) and buffer B, ACN (1% formic acid). Flow rate was 250 μL/min. Gradient conditions were as follows: 17 min gradient from 1% to 70% B and hold on 70% B for 8 min. Analyses generally lasted for 30 min; an additional 4 min were required for column re-equilibration. HPLC was performed using a Dionex Ultimate 3000 HPLC system (Dionex, Germany) equipped with a pump (DGP 3600M), an auto sampler (WPS-3000T), HCTultra PTM Discovery (Bruker Daltonics, Germany), and an ESI source. The mass spectrometer was suitable for LC–MS full scan measurements and LC–MS/MS experiments. The ESI source was operated in the positive ion mode. MS scan measurements were carried out in the mass range of 0–250 m/z using the ultra-scan mode. Profile Analysis software 2.0 (Bruker Daltonics, Germany) was used to perform the multivariate data analysis.

2.5. TLC

TLC is a chromatographic technique used to separate mixtures. Different compounds in the sample mixture travel at different traveling rates because of the differences in their attraction to the stationary phase and their solubility in the solvent. Separation of components can be adjusted by changing the solvent. TLC has been used to determine the formation of the inclusion complex (Hsu et al., 2013).

Sample solutions of HP-β-CD, AS extract, and AS–HP-β-CD complex were prepared by dissolving 0.09 g of HP-β-CD in 1 mL of water, 0.01 g of AS extract in 1 mL of 95% ethanol, and 0.1 g of AS–HP-β-CD complex in 1 mL of water, respectively. 0.01 g of AS extract and 0.09 g of HP-β-CD dissolved in 1 mL of 95% ethanol was used as the solution of the physical mixture of AS extract and HP-β-CD. Then, 1–4 μL of each sample solution was spotted on the silica gel TLC plate and developed in a mixed solvent (ethanol/ethyl acetate = 11/1.8, v/v). After development, the solvent was evaporated, and the dried plates were illuminated using an ultraviolet (UV) light to trace these samples. For sulfuric acid/heat treatment, the dried plates were immersed in an ethanol solution with 10% sulfuric acid followed by heating for 30 min at 60 °C to form charred blots.

2.6. ^1H NMR

^1H NMR spectra were obtained using a Bruker Avance 600NMR spectrometer using deuterated water (D_2O) or dimethyl sulfoxide (DMSO-d6) as the solvent. The chemical shifts (δ) were presented as ppm and referenced to the residual water signal (4.75 ppm).

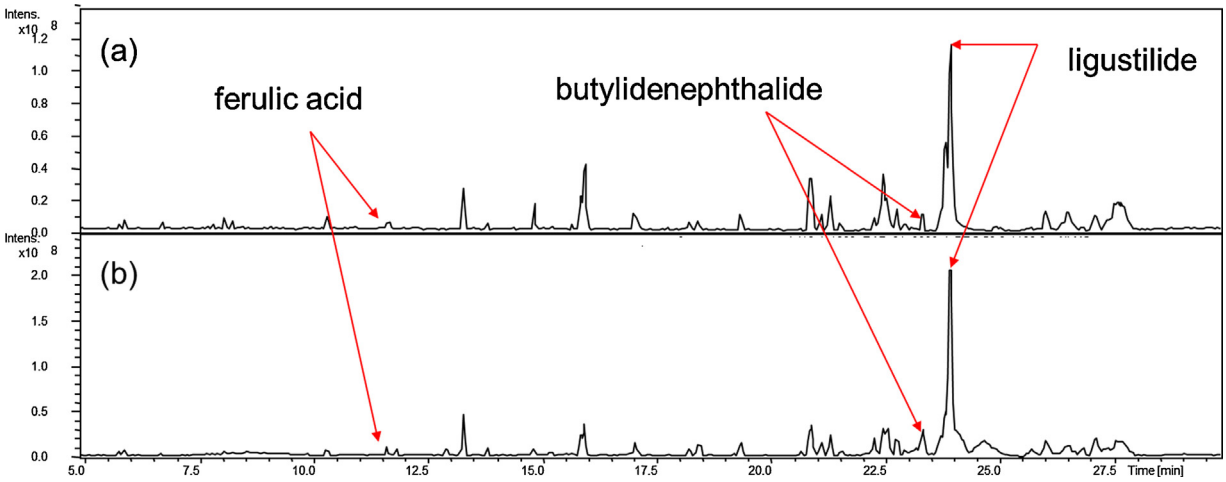

Fig. 2. LC–MS analysis of (a) AS extract and (b) AS–HP-β-CD complex.

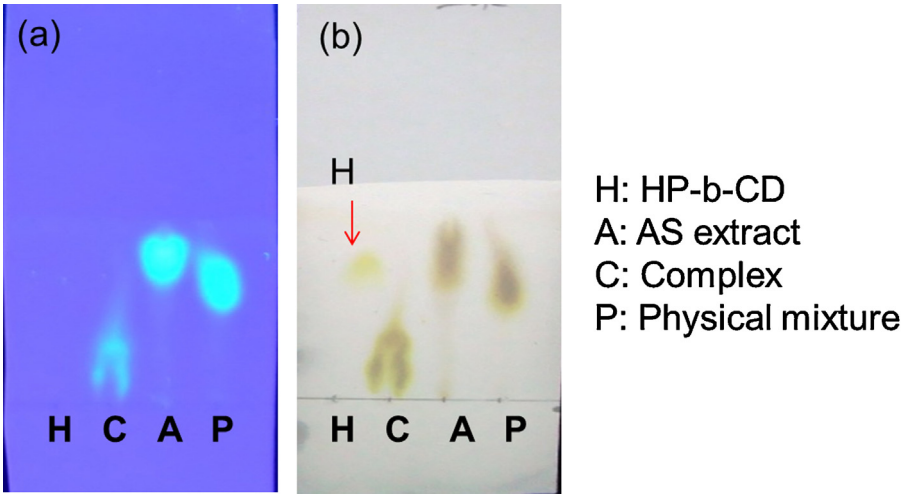

Fig. 3. Thin layer chromatography results of HP-β-CD, AS–HP-β-CD complex, AS extract and the physical mixture (a) under an ultraviolet light and (b) after treatment with sulfuric acid and heat.

2.7. 2D ROESY

2D ROESY is one of the most effective tools for investigating inter- and intra-molecular interactions of guest molecule-CD complex systems. The presence of cross-peaks, which are generated by nuclear Overhauser effects (NOE), between protons of guest molecules and CDs provides useful information about the dynamics and averaged relative inter-/intra-molecular proton distances of these species within 0.4 nm in solution (Ali & Upadhyay, 2008; Jullian, Moyano, Yanez, & Olea-Azar, 2007; Tsai, Tsai, Wu, & Tsai, 2010).

2D ROESY was recorded on a Bruker-600 MHz instrument. The samples were prepared by dissolving 3 mg of the inclusion complex in about 1 mL of D_2O at room temperature. The spin-lock mixing time was set to 500 ms after calibration to ensure the reliability of ROESY cross-peaks.

2.8. Cell culture

Human Hep3B hepatoma cells were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged every 3–4 days.

2.9. MTS assay

The MTS colorimetric assay is used to measure the activity of enzymes that reduce MTS to formazan (a purple dye with an absorbance maximum at 490–500 nm). This assay can be used to determine the cytotoxicity of potential medicinal agents and toxic materials that inhibit cell viability and proliferation. Therefore, we used the MTS assay to measure the proliferation of Hep3B

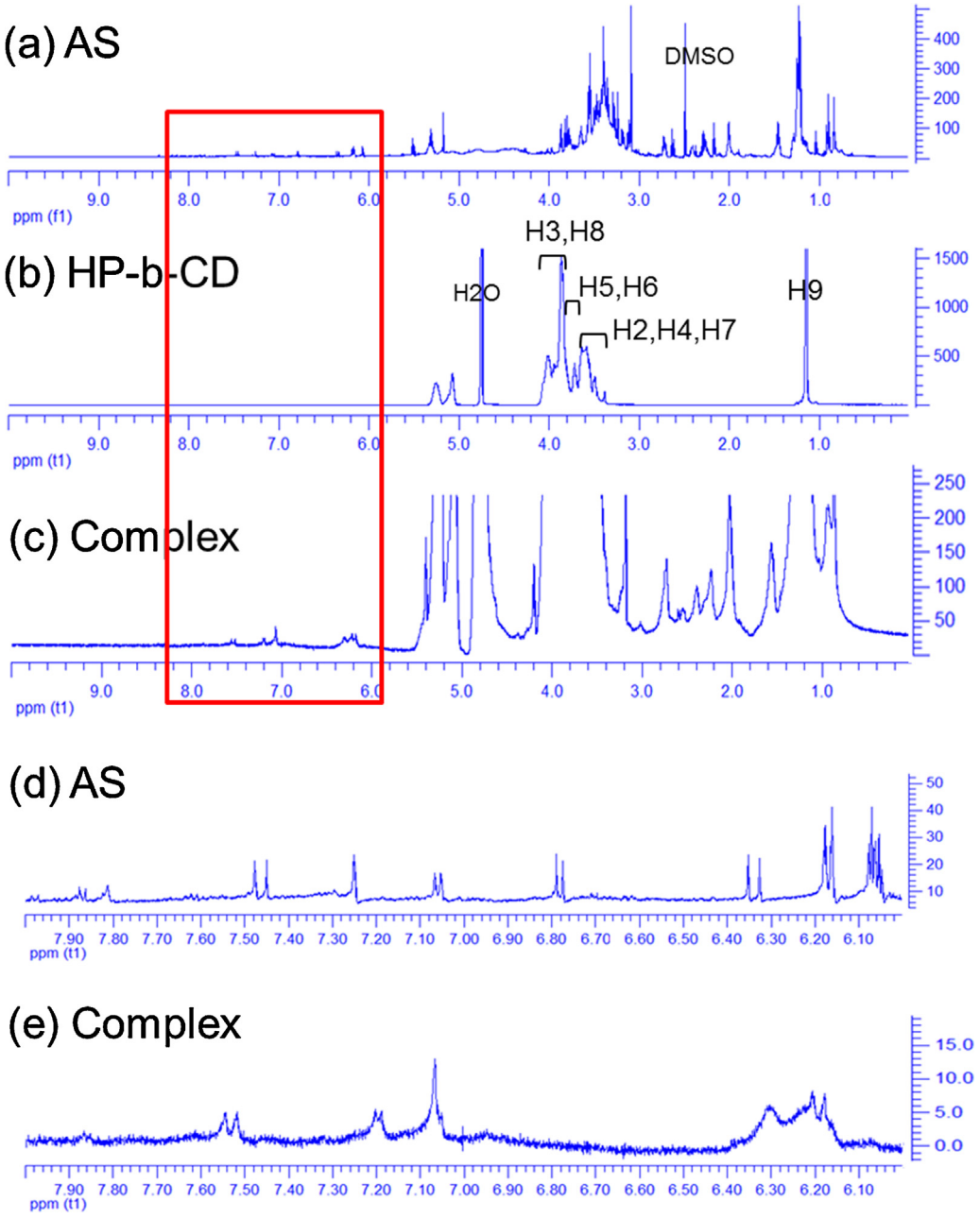

Fig. 4. ^{1}H NMR spectra of (a) AS extract, (b) HP-β-CD, (c) AS–HP-β-CD complex, (d) AS extract (partial spectrum), and (e) AS–HP-β-CD complex (partial spectrum).

hepatoma cells treated with AS extract or AS–HP-β-CD complex for 72 h.

AS extract and AS–HP-β-CD complex were mixed separately in DMEM and added to the cell culture media in doses equivalent to 80 μg/mL, 120 μg/mL, and 160 μg/mL of AS extract. Hep3B cells were cultured in 96-well plates at 5000 cells/well and treated with AS extract and AS–HP-β-CD complex. Cytotoxicity was measured 72 h later by using an MTS cell proliferation assay kit in accordance with the manufacturer's instructions. All experiments were performed in triplicates.

2.10. Cellular uptake

The uptake of HP-β-CD, AS extract, and AS–HP-β-CD complex by Hep3B cells was directly analyzed by confocal laser scanning microscopy (CLSM). Briefly, cells were cultured in 6-well plates at 2×10^4 cells/well. After 24 h, the medium was replaced with fresh medium containing HP-β-CD, AS extract, or AS–HP-β-CD complex for 1 h. The concentrations of HP-β-CD, AS extract, and AS–HP-β-CD complex used were 800 μg/mL, 160 μg/mL, and 960 μg/mL, respectively. The cells were treated with AS extract, and AS–HP-β-CD complex had doses equivalent to 160 μg/mL of AS extract. Cells were washed thrice with PBS to remove the added HP-β-CD, AS extract, or AS–HP-β-CD complex and examined with a CLSM (Leica TCS SP2, Mannheim, Germany). The average fluorescence intensity per cell was determined using IPLab Spectrum version 4.0.8 software (BD Biosciences, San Jose, CA).

2.11. Statistical analysis

All data are expressed as means ± S.D. The statistical analyses were done on the SPSS program (SPSS 15.0; SPSS Institute, Chicago, IL, USA). The two-tailed Student's *t* test was used to examine between AS extract and complex in MTS assay. One-way analysis of variance (ANOVA) with Dunnett's test was used for testing the differences between groups in cellular uptake analysis. A *p*-value of <0.05 was considered significant.

3. Results and discussion

3.1. LC–MS analysis

The LC–MS analyses of AS extract and AS–HP-β-CD complex are shown in Fig. 2(a) and (b), respectively, in which ferulic acid (m/z = 195; retention time = 11.6 min), butylidenephthalide (m/z = 189; retention time = 23.5 min), and ligustilide (m/z = 191; retention time = 23.8 min) are marked. The presence of ferulic acid, butylidenephthalide, and ligustilide in the AS extract indicated that the AS sample used in this study contained the active ingredients described in previous reports (Chao & Lin, 2011; Zhan et al., 2013). Furthermore, there is no significant difference between Fig. 2(a) and (b), suggesting that these active ingredients were retained in the AS–HP-β-CD complex.

3.2. TLC analysis

The TLC results of HP-β-CD, AS–HP-β-CD complex, AS extract and the physical mixture of AS extract and HP-β-CD visualized under a UV light and sulfuric acid/heat treatment are shown in Fig. 3(a) and (b). Because HP-β-CD does not have a fluorescent moiety, no spot was observed under a UV light as shown in Fig. 3(a). However, the AS–HP-β-CD complex, AS extract and the physical mixture of AS extract and HP-β-CD showed fluorescents pots which

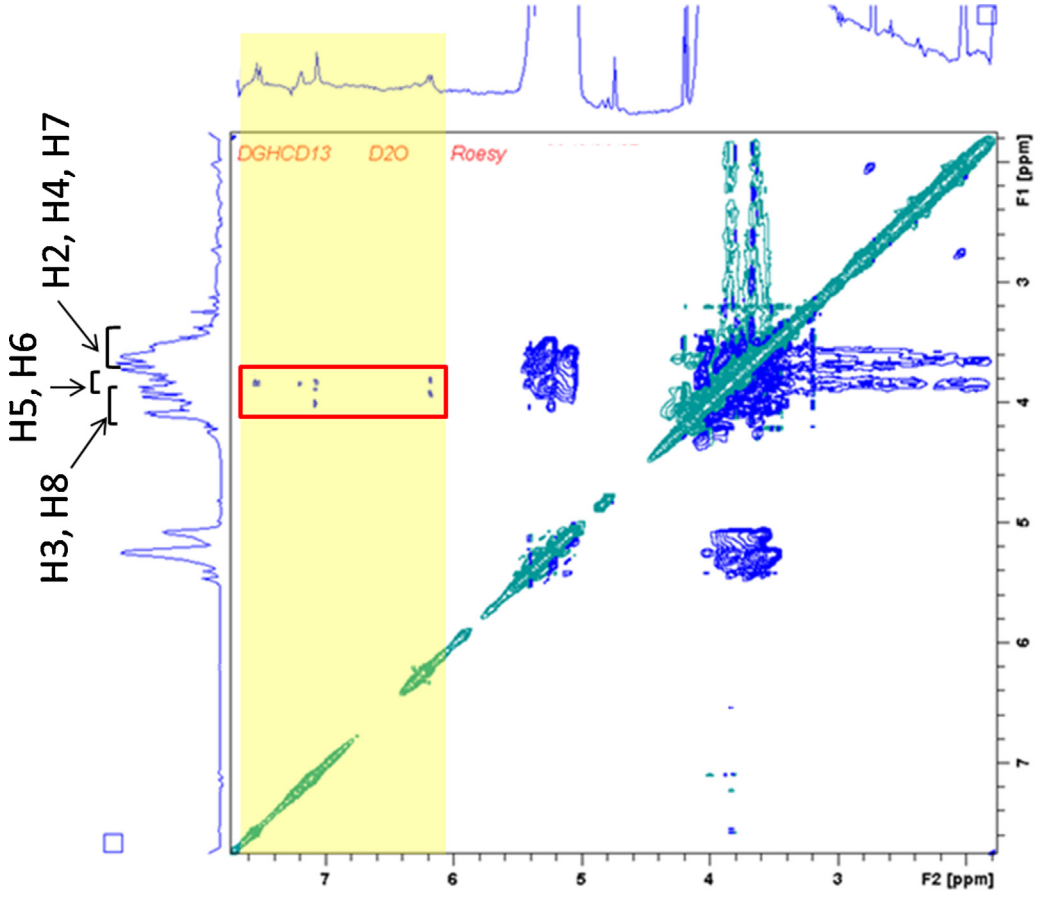

Fig. 5. Two-dimensional rotating frame spectroscopy of AS–HP-β-CD complex.

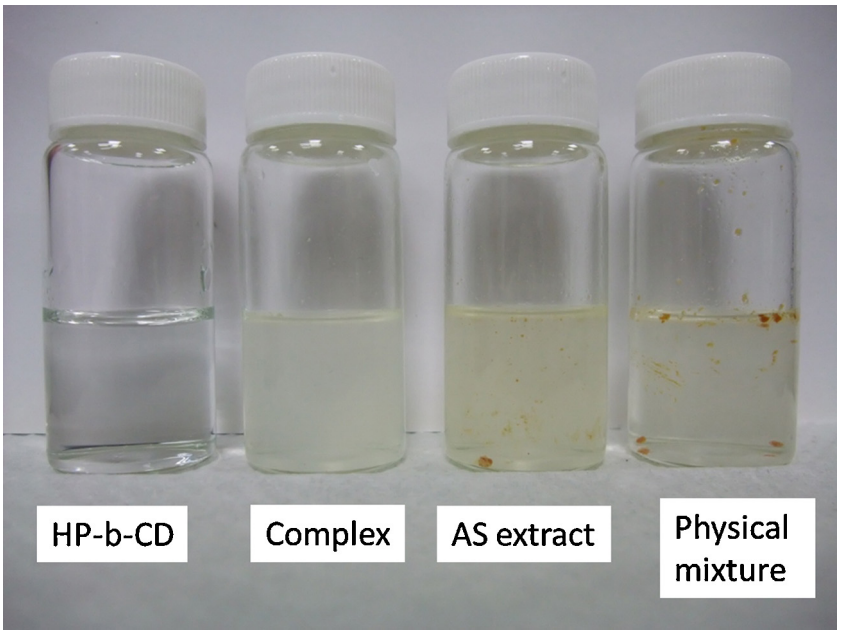

Fig. 6. Images of HP-β-CD, AS–HP-β-CD complex, AS extract, and the physical mixture of HP-β-CD and AS extract in solution state.

were from AS. The AS extract and the physical mixture traveled at a similar rate which was faster than the AS–HP-β-CD complex. The similar traveling rates of AS extract and the physical mixture suggested that AS extract of the physical mixture was not encapsulated by HP-β-CD. The difference in the traveling rates between AS extract and AS–HP-β-CD complex indicated that AS extract had been encapsulated in the CD cavity, and the interaction of AS extract inside AS–HP-β-CD complex with the stationary phase and developing solvent was different from that of AS extract without inclusion.

HP-β-CD became visible after sulfuric acid/heat treatment to form charred blots as shown in Fig. 3(b). Fig. 3(b) shows HP-β-CD and HP-β-CD of the physical mixture which had free HP-β-CD (no AS extract molecules inside) had similar traveling rate. However, the AS–HP-β-CD complex had a shorter traveling distance than them. Those observations suggested that the complex was heavier than free HP-β-CD, which made the complex travel less slowly than free HP-β-CD.

3.3. ^{1}H NMR spectroscopic analysis

Fig. 4(a)–(c) present the ^{1}H NMR spectra of AS extract, HP-β-CD, and the AS–HP-β-CD complex, respectively. The proton resonances of HP-β-CD were assigned on the basis of the relevant literature (Axel Castelli et al., 2008; Rezende et al., 2009). The protons H1–H9 of HP-β-CD were shown and assigned in Fig. 1 (b). Chemical shifts in the 3.85–4.10 ppm range corresponded to H3 and H8; those in the 3.40–3.75 ppm range to H2, H4, and H7; and those in the 3.70–3.85 ppm range to H5 and H6, as shown in Fig. 4(b). The chemical shifts observed in Fig. 4(c) contained elements of those shown in Fig. 4(a) and (b), indicating that the AS–HP-β-CD complex contained both AS extract and HP-β-CD, which was consistent with the LC–MS results.

^{1}H NMR spectroscopy also provided direct evidence for the inclusion of the guest molecule in the HP-β-CD cavity. It is predicted that if inclusion occurs, the physical or chemical environment of the guest molecule will be affected by hydrogen atoms on the internal surface of the cavity (H3 and H5 from any glucose unit of CD (Fig. 1b)). Therefore, changes in the chemical displacement of guest molecule protons were expected (Tsao et al., 2010, 2012). Fig. 4(d) and (e) show the partial ^{1}H NMR spectra of AS extract and AS–HP-β-CD complex, respectively. According to a previous report (Leon, Chavez, & Delgado, 2011), chemical shifts in the range of 6.00–6.20, 6.70–7.10, and 7.85–7.90 ppm could be interpreted as the vinyl hydrogens of ligustilide, aromatic hydrogens of ferulic acid or its derivatives, and aromatic hydrogens of butylidenephthalide, respectively (Fig. 4(d)). We observed changes in the chemical displacement, which indicated that the corresponding molecules were included by HP-β-CD (Fig. 4(d) and (e)).

3.4. 2D ROESY

Fig. 5 presents 2D ROESY spectrum of the AS–HP-β-CD complex. No cross-peak was found between the vinyl protons/aromatic protons (6–8 ppm) of AS extract and H2/H4/H7 (3.40–3.75 ppm), the external protons of HP-β-CD, indicating AS extract did not have any interaction with the outer surface of HP-β-CD. However, they interacted with H5/H6 (3.70–3.85 ppm) and H3/H8 (3.85–4.10 ppm). Because AS extract showed no interaction with the outer surface of HP-β-CD, the cross-peak between AS extract and H3/H8 was caused by H3, not H8. These results suggested that AS extract interacted

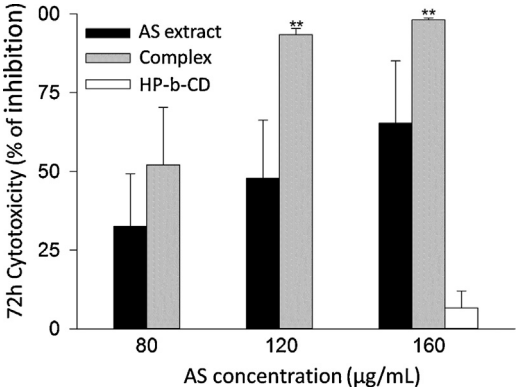

Fig. 7. Cytotoxicity of HP-β-CD, AS extract, and AS–HP-β-CD complex in Hep3B cells treated for 72 h (** indicates $p < 0.01$ between AS extract and complex by t-test).

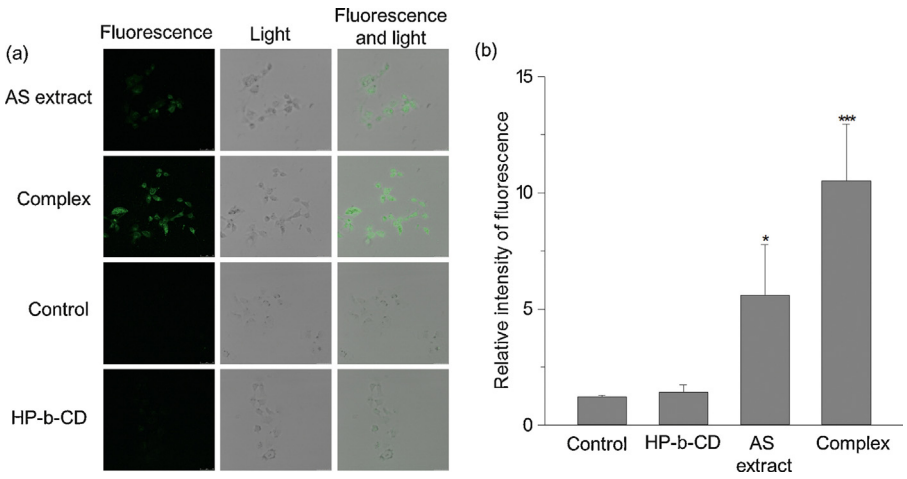

Fig. 8. (a) Fluorescence images and (b) relative fluorescence intensities of Hep3B cells treated with HP-β-CD, AS extract, or AS–HP-β-CD complex for 1 h (* indicates $p < 0.05$ and *** indicates $p < 0.001$ compared to control by the one-way ANOVA).

with internal protons of HP-β-CD (H3, H5 and H6), indicating AS extract was included by HP-β-CD.

3.5. Solubility

Because the AS extract was a mixture, its solubility could not be determined quantitatively. In this study, the solubility test was performed by comparing the solution states of different samples qualitatively. Fig. 6 shows the solution state of AS–HP-β-CD complex (30 mg/mL ddH_2O), HP-β-CD (25 mg/mL ddH_2O), AS extract (5 mg/mL ddH_2O), and the physical mixture of AS extract (5 mg/mL ddH_2O) and HP-β-CD (25 mg/mL ddH_2O). HP-β-CD was dissolved in water, and the AS–HP-β-CD complex was well dispersed. However, AS extract and the physical mixture of AS extract and HP-β-CD appeared in the oil layer and precipitate phases. These results indicated that the AS extract of AS–HP-β-CD complex had been included by HP-β-CD, and consequently, its immediate aqueous solubility/dispersibility had been improved.

3.6. Cytotoxicity

The 72 h cytotoxicity results at doses equivalent to 80, 120, and 160 μg/mL of AS extract and 160 μg/mL of HP-β-CD are shown in Fig. 7. The data indicated that the cytotoxic effect of HP-beta-CD on the Hep3B cells was negligible. However, AS extract and AS–HP-β-CD complex inhibited cell proliferation in a dose-dependent manner, significantly. These results were consistent with those reported in previous studies (Chen et al., 2008; Yu et al., 2010).

The cytotoxic effect of the AS–HP-β-CD complex on Hep3B cells was greater than that of the AS extract at equivalent doses. The increased cytotoxicity associated with the complex suggested more efficient cellular uptake of AS molecules from the AS–HP-β-CD complex than from the AS extract alone. This could be due to the enhanced aqueous solubility of AS molecules when they were complexed with HP-β-CD (Brewster & Loftsson, 2007). Other hydrophobic drugs, such as curcumin (Yallapu, Jaggi, & Chauhan, 2010), dichloro(dipyridine)platinum (Horvath et al., 2008), and pyrazolopyrimidines (Dreassi et al., 2010) have also shown enhanced cellular uptake when complexed with CDs, which enhanced their cytotoxic effect.

3.7. Cellular uptake

The uptake of HP-β-CD, AS extract and AS–HP-β-CD complex, containing equivalent AS doses (160 μg/mL), by Hep3B cells after treatment for 1 h was analyzed by CLSM. The fluorescence, optical, and merged microscopic images of these samples are shown in Fig. 8(a). No fluorescence was detected in control and HP-β-CD-treated cells. However, the fluorescence intensity was higher in cells treated with the AS–HP-β-CD complex as compared to that of cells treated with the AS extract. The average fluorescence intensity per cell (Fig. 8b) was determined (Yallapu et al., 2010). These data suggest that HP-β-CD increased AS extract uptake by Hep3B cells, which is consistent with the results of cytotoxicity analysis.

4. Conclusions

In this study, an AS–HP-β-CD complex was prepared from an ethanolic extract of AS and HP-β-CD by freeze-drying method. LC–MS results suggested both AS extract and AS–HP-β-CD complex had the active ingredients, ferulic acid, butylidenephthalide, and ligustilide. TLC, ^{1}H NMR spectroscopy and 2D ROSEY results showed that insoluble AS extract was included by HP-β-CD to form the AS–HP-β-CD complex. The AS–HP-β-CD complex had higher aqueous solubility/dispersibility than AS extract alone, which improved the uptake of AS extract by hepatoma cells, and thus, increased the cytotoxicity of AS. These results indicated that complexing AS extract with HP-β-CD enhances the effect of AS on Hep3B cells by increasing its water solubility/dispersibility and cellular uptake. These observations suggested that the use of HP-β-CD in stabilizing AS extract was possible for herbal medicine application in the future.

Acknowledgments

The authors would like to thank Prof. Lei Wan at China Medical University for cell culture technique support. This work was partially supported by grants from China Medical University (CMU101-S-06 and CMU102-S-25). Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center and mass spectrometric analyses were performed by the Proteomics Research Core Laboratory, Office of Research & Development at China Medical University.

References

Ali, S. M., & Upadhyay, S. K. (2008). Complexation study of midazolam hydrochloride with β-cyclodextrin: NMR spectroscopic study in solution. *Magnetic Resonance in Chemistry*, *46*, 676–679.

Andreu-Sevilla, A. J., Carbonell-Barrachina, A. A., Lopez-Nicolas, J. M., & Garcia-Carmona, F. (2011). Sensory quality, volatile compounds and color of pear juice

treated with beta-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *70*, 453–460.
Axel Castelli, V. V., Trivieri, G., Zucchelli, I., Brambilla, L., Barbuzzi, T., Castiglioni, C., et al. (2008). Characterisation of an inclusion complex between cladribine and 2-hydroxypropyl-*b*-cyclodextrin. *Journal of Pharmaceutical Sciences*, *97*, 3897–3906.
Ayala-Zavala, J. F., Soto-Valdez, H., Gonzalaz-Leon, A., Alvarez-Parrilla, E., Martin-Belloso, O., & Gonzalez-Aguilar, G. A. (2008). Microencapsulation of cinnamon leaf (*Cinnamomum zeylanicum*) and garlic (*Allium sativum*) oils in beta-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *60*, 359–368.
Brewster, M. E., & Loftsson, T. (2007). Cyclodextrins as pharmaccutical solubilizers. *Advanced Drug Delivery Reviews*, *59*, 645–666.
Chao, W. W., & Lin, B. F. (2011). Bioactivities of major constituents isolated from *Angelica sinensis* (Danggui). *Chinese Medicine*, *6*, 29.
Chen, Y., Jian, M. H., Lin, C. C., Kang, J. C., Chen, S. P., Lin, P. C., et al. (2008). The induction of orphan nuclear receptor Nur77 expression by *n*-butylenephthalide as pharmaceuticals on hepatocellular carcinoma cell therapy. *Molecular Pharmacology*, *74*, 1046–1058.
Dreassi, E., Zizzari, A. T., Mori, M., Filippi, I., Belfiore, A., Naldini, A., et al. (2010). 2-Hydroxypropyl-beta-cyclodextrin strongly improves water solubility andanti-proliferative activity of pyrazolo[3,4-ɒ]pyrimidines Src-Abl dual inhibitors. *European Journal of Medicinal Chemistry*, *45*, 5958–5964.
Feng, Q., Ren, Y., Wang, Y., Ma, H., Xu, J., Zhou, C., et al. (2008). Anti-inflammatory effect of SQC-beta-CD on lipopolysaccharide-induced acute lung injury. *Journal of Ethnopharmacology*, *118*, 51–58.
Gould, S., & Scott, R. C. (2005). 2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): A toxicology review. *Food and Chemical Toxicology*, *43*, 1451–1459.
Horvath, G., Premkumar, T., Boztas, A., Lee, E., Jon, S., & Geckeler, K. E. (2008). Supramolecular nanoencapsulation as a tool: Solubilization of the anticancer drug trans-dichloro(dipyridine)platinum(II) by complexation with beta-cyclodextrin. *Molecular Pharmaceutics*, *5*, 358–363.
Hsu, C., Yu, S., Tsai, F., & Tsai, Y. (2013). Enhancement of rhubarb extract solubility and bioactivity by 2-hydroxypropyl—Cyclodextrin. *Carbohydrate Polymers*, *98*, 1422–1429.
Jullian, C., Moyano, L., Yanez, C., & Olea-Azar, C. (2007). Complexation of quercetin with three kinds of cyclodextrins: An antioxidant study. *Spectrochimica Acta, A: Molecular and Biomolecular Spectroscopy*, *67*, 230–234.
Kalogeropoulos, N., Yannakopoulou, K., Gioxari, A., Chiou, A., & Makris, D. P. (2010). Polyphenol characterization and encapsulation in beta-cyclodextrin of a flavonoid-rich *Hypericum perforatum* (St John's wort) extract. *LWT—Food Science and Technology*, *43*, 882–889.
Leon, A., Chavez, M. I., & Delgado, G. (2011). ^{1}H and DOSY NMR spectroscopy analysis of *Ligusticum porteri* rhizome extracts. *Magnetic Resonance in Chemistry*, *49*, 469–476.
Michali, I., Karathanos, V. T., Chiou, A., Karathanos, V. T., & Mourtzinos, I. (2008). Thermal stability of anthocyanin extract of *Hibiscus sabdariffa* L. in the presence of beta-cyclodextrin. *Journal of Agricultural and Food Chemistry*, *56*, 10303–10310.
Mourtzinos, I., Salta, F., Yannakopoulou, K., Chiou, A., & Karathanos, V. T. (2007). Encapsulation of olive leaf extract in beta-cyclodextrin. *Journal of Agricultural and Food Chemistry*, *55*, 8088–8094.
Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians*, *55*, 74–108.
Rezende, K. R., Soares, L. A., Leal, A. F. V. B., Fraceto, L. F., Maia, E. R., Resck, I. S., et al. (2009). Host–guest system of 4-nerolidylcatechol in 2-hydroxypropyl-beta-cyclodextrin: preparation, characterization and molecular modeling. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *64*, 23–35.
Tsai, Y., Tsai, H. H., Wu, C. P., & Tsai, F. J. (2010). Preparation, characterisation and activity of the inclusion complex of paeonol with β-cyclodextrin. *Food Chemistry*, *120*, 837–841.
Tsao, J. Y., Wu, C. P., Tsai, H. H., Peng, K. C., Lin, P. Y., Su, S. Y., et al. (2012). Effect of hydroxypropyl-beta-cyclodextrin complexation on the aqueous solubility, structure, thermal stability, antioxidant activity, and tyrosinase inhibition of paeonol. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *72*, 405–411.
Tsao, J. Y., Tsai, H. H., Wu, C. P., Lin, P. Y., Su, S. Y., Chen, L. D., et al. (2010). Release of paeonol-beta-CD complex from thermo-sensitive poly(*N*-isopropylacrylamide) hydrogels. *International Journal of Pharmaceutics*, *402*, 123–128.
Vyas, A., Saraf, S., & Saraf, S. (2008). Cyclodextrin based novel drug delivery systems. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *62*, 23–42.
Wei, S. Y., Xu, C. J., Mok, D. K., Cao, H., Lau, T. Y., & Chau, F. T. (2008). Analytical comparison of different parts of Radix Angelicae Sinensis by gas chromatography coupled with mass spectrometry. *Journal of Chromatography A*, *1187*, 232–238.
Yallapu, M. M., Jaggi, M., & Chauhan, S. C. (2010). Beta-cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells. *Colloids and Surfaces B: Biointerfaces*, *79*, 113–125.
Yi, L., Liang, Y., Wu, H., & Yuan, D. (2009). The analysis of radix Angelicae Sinensis (Danggui). *Journal of Chromatography A*, *1216*, 1991–2001.
Yu, Y., Yu, S., Su, K., Wei, C., Jian, M., Lin, P., et al. (2010). Extended O6-methylguanine methyltransferase promoter hypermethylation following *n*-butylidenephthalide combined with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on inhibition of human hepatocellular carcinoma cell growth. *Journal of Agricultural and Food Chemistry*, *58*, 1630–1638.
Zhan, J. Y., Zhang, W. L., Zheng, K. Y., Zhu, K. Y., Chen, J., Chan, P., et al. (2013). Chemical changes of Angelicae Sinensis Radix and Chuanxiong Rhizoma by wine treatment: chemical profiling and marker selection by gas chromatography coupled with triple quadrupole mass spectrometry. *Chinese Medicine*, *8*, 12.