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Bioactivity-guided fractionation of the volatile oil of *Angelica sinensis radix* designed to preserve the synergistic effects of the mixture followed by identification of the active principles

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ABSTRACT

In natural product research, it is a common experience that fractionation of biologically-active crude extracts can lead to the loss of their original activity. This is attributed to synergistic effects, where two or more components are required to be present together for full activity of the sample. Our previous study showed that a volatile oil of *Angelica sinensis radix* (VOAS) inhibited endothelial cell proliferation in culture. Here we have used a bioactivity-guided fractionation method to preserve any synergistic effects of VOAS combining countercurrent chromatography (CCC), the MTS cell viability assay and gas chromatography (GC). Using a two-phase CCC solvent system (heptane–ethyl acetate–methanol–water at a volume ratio of 27:23:27:23%), forty-five fractions were isolated, nine of which exhibited anti-endothelial properties. GC analysis showed two bioactive alkylphthalides, *Z*-ligustilide and *n*-butylidenephthalide (BP) were the major compounds detected in the bioactive fractions, and were absent in non-bioactive fractions. Our results indicate that *Z*-ligustilide and BP are the main constituents responsible for the anti-endothelial properties of VOAS. This rapid and reliable approach in preserving sample activity while isolating and identifying its active compounds suggests that this protocol can be a powerful tool for drug discovery from natural products.

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1. Introduction

Angelica sinensis radix (Apiaceae/Umbelliferae), also known as danggui or dongquai, is one of the most commonly used traditional Chinese medicines. This medicinal herb is mainly applied with others in composite formulae for the treatment of cardiovascular diseases, anemia and gynecological dysfunction in Asia, and is often referred to as 'female ginseng'.

The major constituents of *A. sinensis radix* associated with biological activity are thought to be alkylphthalides, coumarins, polysaccharides and ferulic acid [1]. Alkylphthalides are present in the volatile oil of *A. sinensis radix*, whereas polysaccharides and ferulic acid are mainly detected in the aqueous extracts. *Z*-ligustilide (Fig. 1A) is the most abundant alkylphthalide in the volatile oil of *A. sinensis radix* and is often used as a biomarker in the quality assessment of *A. sinensis radix* [2–4]. In early in vitro and in vivo studies, *Z*-ligustilide has been shown to be bioactive [5–8]. *n*-Butylidenephthalide (BP), another alkylphthalide also present in the volatile oil, has a very similar chemical structure to *Z*-ligustilide (Fig. 1B). These two compounds are often studied simultaneously for their chemical and biological properties [9–12].

Countercurrent chromatography (CCC) is a support-free liquid chromatographic technique in which the solute separation is based on partitioning between two immiscible liquid phases. With both the mobile and the stationary phases being liquid, CCC always obtains high sample recovery [13]. Thus all the components in a mixture will elute with no danger of irreversible adsorption or catalytic degradation of some components of interest, as may be the case with solid phase chromatographic techniques. In addition, CCC

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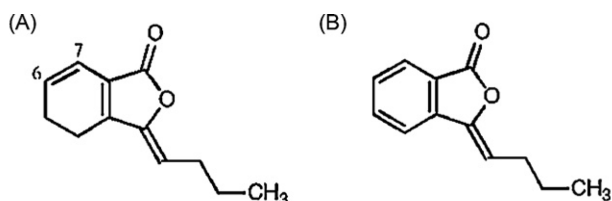

Fig. 1. Chemical structures of (A) *Z*-ligustilide and (B) *n*-butylidenephthalide.

permits a crude sample to be introduced directly into the column, and has been applied extensively for the separation and purification of natural products [13]. To date, only one group has reported the use of CCC in studying *A. sinensis radix*, and this was for the separation and purification of *Z*-ligustilide from a crude carbon dioxide supercritical fluid extract of *A. sinensis radix* [4]. They reported a total of 38 mg *Z*-ligustilide at 98.8% purity was obtained in one step from 200 mg crude extract.

We had previously found that a volatile oil of *A. sinensis radix* (VOAS) exhibited inhibitory effects on endothelial cell proliferation in vitro [14]. In the present study, we aim to determine the chemical identity of bioactive components in VOAS using a bioactivity-guided fractionation method combining a robotic solvent selection system, countercurrent chromatography (CCC) separation, the MTS cell viability assay and gas chromatography (GC) analysis. The bioactivity measurement was targeted at the anti-endothelial action of the test material.

2. Experimental

2.1. Materials and chemicals

All solvents used in the solvent selection process and CCC purification runs were either analytical or HPLC grade purchased from Fisher Scientific Ltd, Loughborough, UK. BP was purchased from Lancaster Synthesis Ltd (Newgate, Morecambe, UK). *Z*-ligustilide was isolated from roots of *A. sinensis* (Oliv.) Diels by sonication extraction, followed by HPLC separation as described by Li et al. [3] (see Supplementary Data). VOAS was extracted from raw *A. sinensis radix* material using 95% ethanol, followed by solvent and chromatographic separation and purification. The raw *A. sinensis radix* material was purchased from Good Agricultural & Collection Practice (GACP) site in Gansu Province, China. The chemical composition of VOAS was determined by GC–MS analysis and was shown in our previous publication: VOAS contained 51 identifiable compounds, of which the relative proportion of *Z*-ligustilide and BP were 15.0% and 2.5%, respectively [14].

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained at elective Caesarean sections performed at the Rosie Hospital in Cambridge, UK. Umbilical cords were collected with Ethics Committee approval and written informed patient consent. HUVECs were grown in endothelial cell basal medium supplemented with EGM-2 (Endothelial Cell Growth Medium-2) SingleQuots (Lonza, Slough, UK). Cells were maintained at 37 °C in 5% CO_2 humidified tissue culture incubator. All test chemicals and VOAS materials were dissolved in vehicle dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Dorset, UK) for in vitro experiments.

2.2. Apparatus

The solvent selection process was performed by a Perkin Elmer Multiprobe II liquid handling robot fitted with 4 probes and 1 mL syringes [15].

The CCC instrument used was a Mini CCC commercially available from Dynamic Extractions Ltd, Slough, UK. The instrument was equipped with a coil of 17.2 mL volume and 0.8 mm bore. The extra coil volume was 0.39 mL and β range 0.68–0.79 with a rotor radius R = 50 mm. The rotor was spun at 2000 rpm (approximately 220 × g). The tubing material was Teflon PFA (perfluoroalkoxy).

The GC device was an Agilent 6890N equipped with Agilent 7683 autoinjector. The column was an HP-5 from J&W, 30 m × 0.32 mm bore with 0.25 μm film thickness. Carrier gas was hydrogen supplied by a Domnick Hunter 40H generator. Detection was both thermal conductivity detection (TCD) and flame ionization detection (FID) although only the data from the FID was used for this project. The FID was supplied with zero air and nitrogen make-up gas from a Domnick Hunter G6010E compressor and nitrogen generator plus a Domnick Hunter Nitrox 10-ZA zero air catalytic converter.

2.3. Preparation of two-phase solvent systems and sample solutions

Thirty-six solvent systems (Table 1) over a wide range of solvent polarities were made in 4 mL volume each in glass test tubes by an automated liquid handling robot. Test sample VOAS (80 mg) was added to 4 mL solvent systems and mixed thoroughly. After phase separation, both upper and lower phases were collected. Serial dilutions were made using cell culture medium and subjected to bioassay. The selected solvent systems for CCC fractionation were prepared in a separating funnel by adding the component solvents in the required volume ratios. After the contents have reached room temperature, they were mixed thoroughly and the phases allowed to separate. The upper and lower phases were then collected and stored separately for use as mobile and stationary phase, respectively. The injection sample solution was prepared by dissolving 10 μL (8 mg) VOAS in 1 mL mobile phase before fractionation.

2.4. CCC separation procedure

The CCC tubing was first filled with the stationary phase (lower phase) with no rotation. Then the coil was rotated at 1900 rpm (610 × g) as the mobile phase (upper phase) was pumped into the coil at a flow rate of 0.5 mL/min. Stationary phase was initially displaced until hydrodynamic equilibrium was established and mobile phase eluted. The spin speed was increased up to 2000 rpm (670 × g) to improve retention of the stationary phase, and sample solution was injected into the coil through the injection valve. CCC fractions, 1 mL each, were then collected and solvent was removed by evaporation using a SpeedVac (30 °C, 1500 rpm). The fractions were subsequently resuspended in 20 μL DMSO and subjected to the MTS assay and GC analysis.

2.5. GC analysis and identification of CCC fractions

GC was used for the detection of chemical substances in CCC fractions. The conditions of GC are listed in Table 2. *Z*-ligustilide and BP both dissolved in methanol were used as pure compound standards.

2.6. MTS cell viability assay

Cell viability was estimated by the MTS [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, UK). Briefly, cells were plated at a density of 8×10^3 cells/well in 96-well plates for 24 h followed by the addition of various concentrations of test samples. After 48 h of incubation, 20 μL of CellTiter 96® AQ_{ueous} One Solution Reagent was added into each well and the plates were incubated for a further 2 h without exposing to light. The absorbance at 492 nm was

Table 1
Robotic solvent system selection. Numbers in the table indicate the volume ratio (%) of each solvent. Total volume of each solvent system = 4 mL.

No	Heptane	Ethyl acetate	Methanol	Butanol	Water	Acetonitrile	Toluene	Acetic acid	Acetone	*tert*-Butyl methyl ether	Dichloromethane	Ethanol	Propanol
1				50.0	50.0								
2		20.0		30.0	50.0								
3		40.0		10.0	50.0								
4		50.0			50.0								
5	7.3	42.8	7.3		42.8								
6	12.5	37.5	12.5		37.5								
7	20.0	30.0	20.0		30.0								
8	27.3	22.8	27.3		22.8								
9	35.8	14.3	35.8		14.3								
10	41.8	8.3	41.8		8.3								
11	47.5	2.5	47.5		2.5								
12	50.0		50.0										
13	50.0		45.0			5.0							
14	50.0		30.0			20.0							
15	50.0					50.0							
16	50.0					45.0	5.0						
17	50.0					30.0	20.0						
18				47.5	47.5			5.0					
19				45.0	45.0			10.0					
20	40.0		28.0		12.0						20.0		
21				20.0	50.0	10.0				20.0			
22	31.3	12.5	25.0			31.3							
23				42.1	52.6				5.3				
24	16.7	16.7			16.7				50				
25	50.0		40.0						10.0				
26				20.0	50.0	10.0				20.0			
27					50.0					50.0			
28			40.0		26.7						33.3		
29	50.0					40.0					10.0		
30	37.5				31.3							31.3	
31	25.7	25.7			15.4							33.3	
32		40.0			40.0							20.0	
33		25.0		40.0	30.0							5.0	
34				44.5	44.5							11.1	
35	50.0				37.5								12.5
36	57.2		22.9		5.7								14.3

measured using a 96-well plate reader. Data were expressed as the absorbance percentage of medium control and are means ± SD. All experiments were performed in triplicate. Statistical significance was analyzed using unpaired Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Determination of an optimum CCC solvent system

Countercurrent chromatography was chosen as a suitable purification method for this application since, being a liquid–liquid technique, there is no loss of components from irreversible absorption. In classical CCC purifications, a suitable solvent system is selected according to the distribution ratio (D) of a target compound in the solvent system. D should be within the approximate range 0.2–5 and preferably around 1.0 [15]. In this research, a solvent system was identified on the basis of the biological activity eluting at an acceptable time in the chromatographic process, regardless of whether it was one or several components eluting at that time.

To maximize the fractionation of active principles, a robotic solvent system selection was applied, covering a wide range of polarities and solvent combinations (Table 1). The robotic handling of the solvent systems meant that much larger numbers could be assessed, increasing the chances of finding one that contained the activity in a narrow band. VOAS was dissolved in all thirty-six phases systems and bioactivity measurements made on both the upper and lower phases. Both phases of systems 8 (heptane–ethyl acetate–methanol–water at a volume ratio of 27:23:27:23%) and 32 (ethyl acetate–water–ethanol at a volume ratio of 40:40:20%) produced similar and relatively strong anti-endothelial effects (Fig. 2), indicating acceptable distribution ratios for CCC.

Table 2
Gas chromatography conditions.

Gas chromatograph:	Agilent 6890N
Column:	J&W HP-5, part No 19091J-413
Column dimensions:	30 m length, 0.32 mm bore, 0.25 μm film
Oven initial temp.:	40 °C
Oven temperature ramp:	15 °C/min up to 170 °C then 20 °C/min to 310 °C held at 310 °C for 4 min
Total run time:	20 min
Injector temp.:	250 °C
Injection volume:	2 μL
Split ratio:	10:1 (split flow 25.0 mL/min)
Carrier gas:	Hydrogen, 5.69 psi constant pressure
Carrier gas velocity:	45 cm/s average
Detector:	Flame ionization, 325 °C
Detector gases:	Hydrogen 40 mL/min, air 450 mL/min, nitrogen 50 mL/min

3.2. Bioactivity-guided fractionation using CCC

Using solvent system 8 acceptable stationary phase retention was obtained (79%). Forty-five fractions were separated by CCC and subjected to the MTS cell viability study. To reduce the amount of assay testing, three consecutive fractions were combined into one fraction group, i.e. fraction group I = fractions 1–3. Fig. 3A demonstrates that fraction groups III–VI at 1:100 dilution exhibited

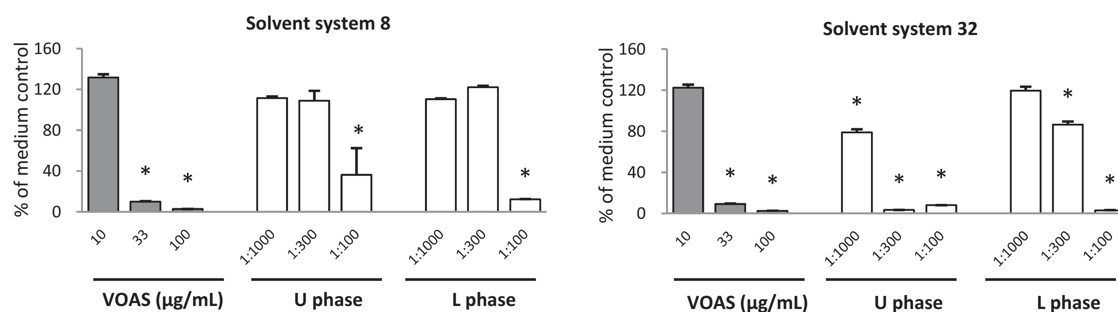

Fig. 2. Bioactivity-guided selection of an ideal solvent system for the fractionation of VOAS on CCC. HUVECs were treated with a serial dilution (1:100, 1:300 and 1:1000) of solvent systems contained VOAS. After 48 h of incubation, MTS assay was performed to determine cell viability. Data are expressed as % of medium control, showing mean ± SD. *Significant inhibitory effects on HUVEC viability compared to medium control ($P < 0.05$, unpaired Student's *t*-test). VOAS at 10, 33 and 100 μg/mL was used as an inhibitory control. Both upper (U) and lower (L) phases of solvent systems 8 (heptane–ethyl acetate–methanol–water at a volume ratio of 27:23:27:23%) and 32 (ethyl acetate–water–ethanol at a volume ratio of 40:40:20%) exhibited similar and strong anti-endothelial properties.

anti-endothelial properties as compared to vehicle control ($P < 0.05$, unpaired Student's *t*-test). Individual fractions of the active fraction groups, also at 1:100 dilution, were further tested separately on HUVECs. The results showed that nine fractions, fractions 7–15, produced significant inhibitory effects on HUVEC viability ($P < 0.05$, unpaired Student's *t*-test) (Fig. 3B). In addition, the injection sample at 1:100 dilution exerted similar inhibitory actions as VOAS at 33 and 100 μg/mL (data not shown). The residue sample (coil pumped-out) showed no significant effects on HUVECs ($P > 0.05$, unpaired Student's *t*-test) (data not shown).

Solvent system 32 was also tested on CCC. However, the percent stationary phase retention at equilibrium was poor (less than 10%) with most of the remainder of the stationary phase eluting from the column after injection of the sample. Thus a single peak was obtained at a point corresponding to the total coil volume and no separation occurred. Since the phase system appeared unstable under the CCC conditions employed, all further work concentrated on solvent system 8.

3.3. Identification and isolation of the active components in VOAS

We employed GC to investigate the chemical identity of the bioactive components in VOAS. Pure compounds BP and *Z*-ligustilide were used as standards and were eluted at 12.2 min and 12.8 min, respectively (Fig. 4A–C). Fig. 4D demonstrates the GC chromatograph of VOAS in methanol. Besides BP and *Z*-ligustilide, a large number of minor components were detected, consistent with the complexity of components in VOAS as previously described [14].

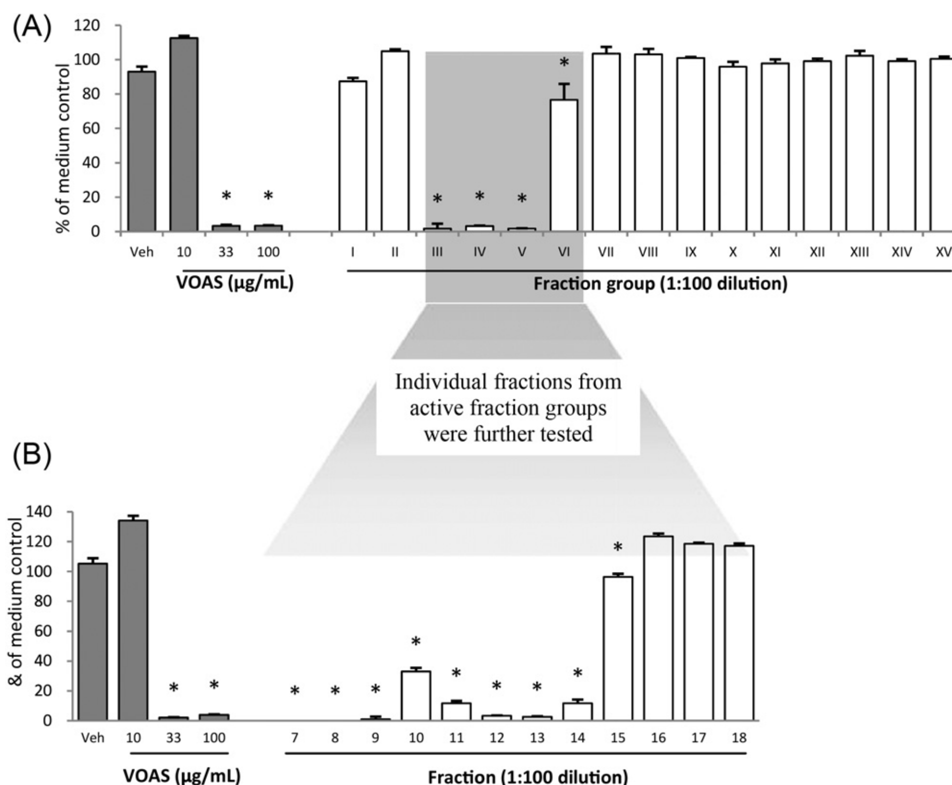

Fig. 3. Effects of VOAS fractions from solvent system 8 (heptane–ethyl acetate–methanol–water at a volume ratio of 27:23:27:23%) on HUVEC viability. HUVECs were treated with (A) VOAS fraction groups (three consecutive fractions combined into one group) at 1:100 dilution and (B) individual fractions at 1:100 dilution from active fraction groups. MTS assay was performed after 48 h of incubation. Data are expressed as % of medium control and are mean ± SD. *Significant inhibitory effects compared to vehicle control (Veh, DMSO 1%) ($P < 0.05$, unpaired Student's *t*-test). VOAS at 10, 33 and 100 μg/mL was used as an inhibitory control. The results demonstrated that fraction groups III–VI and fractions 7–15 exhibited significant anti-endothelial properties.

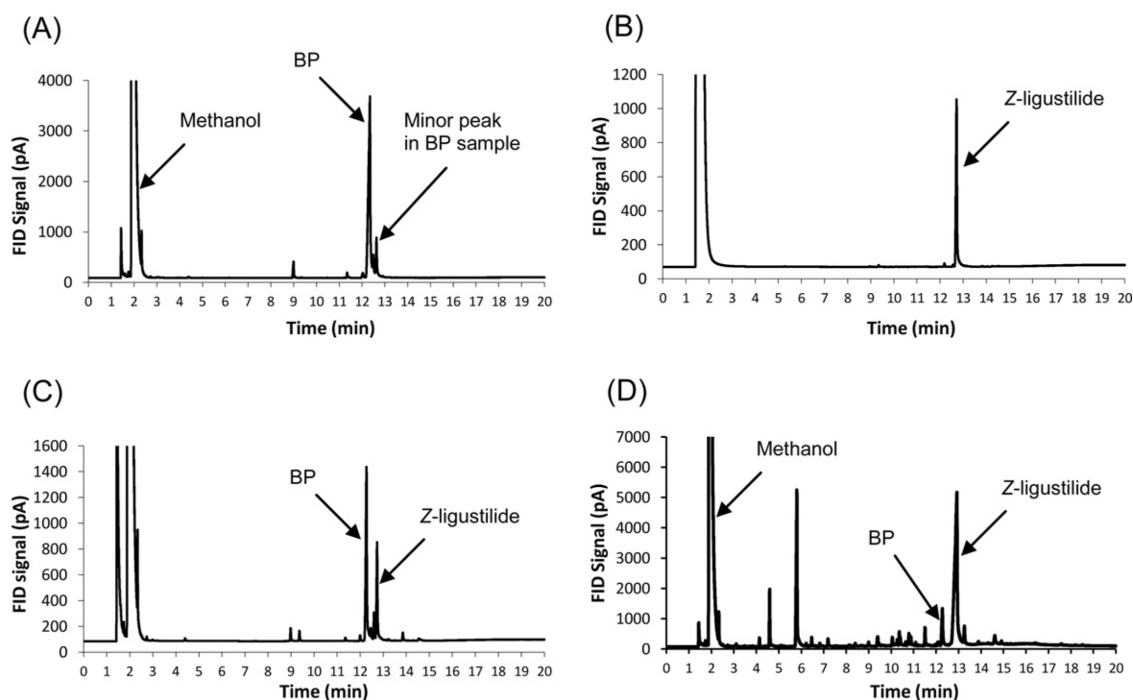

Fig. 4. GC analysis of (A) BP in methanol, (B) *Z*-ligustilide in methanol, (C) BP and *Z*-ligustilide in methanol and (D) VOAS in methanol. GC conditions see Table 2. BP and *Z*-ligustilide were eluted at 12.2 min and 12.8 min, respectively. *Note*: the range of *Y* axis scale is different in each figure.

Interestingly, all the active fractions from solvent system 8 (fractions 7–15) contained *Z*-ligustilide, although some fractions were only with small amounts of *Z*-ligustilide, such as fractions 11 and 14. Fig. 5 shows the GC chromatograms of fractions 7–9 and 11, which also contain two large early peaks which arise from solvents in the phase systems. Moreover, most of the BP was shown to be present in fractions 8 and 9 only (Fig. 5). The peak areas of *Z*-ligustilide and BP in fractions 2–17 were measured and illustrated in Fig. 6B. For the inactive fractions, the only detectable compounds in fractions 1–5 appeared to be solvent peaks, and for fraction 6 additionally a very small blip above baseline (data not shown). Very few peaks were seen in the fractions beyond fraction 19, and a virtually blank baseline was observed from fractions 21 onwards (data not shown). Our results suggest a good correlation between the bioactivities and the presence of *Z*-ligustilide and BP in the fractions.

To further investigate the relationship between the anti-endothelial action and the amount of *Z*-ligustilide and BP in the fractions, we tested a serial dilution of the fractions (1:100, 1:200, 1:1,000 and 1:10,000) on endothelial cells by MTS assay. The MTS results (Fig. 6A) were then compared with the GC data of *Z*-ligustilide and BP (Fig. 6B). Our results indicated a strong correlation between the bioactivity potency and the amount of

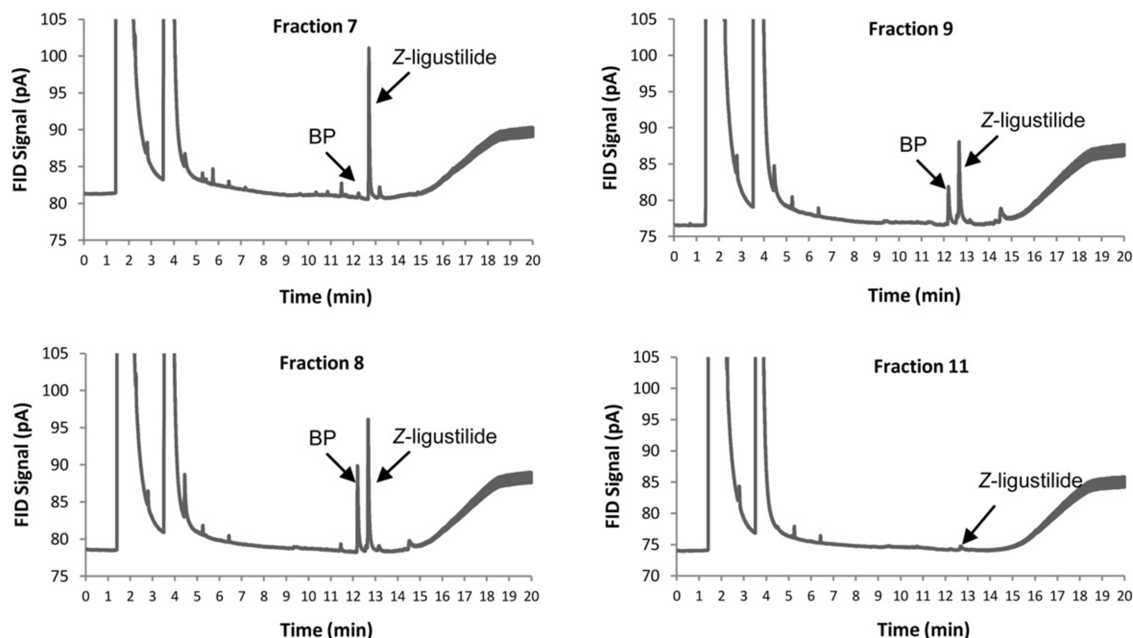

Fig. 5. GC analysis of active VOAS fractions from CCC fractionation in solvent system 8 (heptane–ethyl acetate–methanol–water at a volume ratio of 27:23:27:23%). This figure shows GC chromatograms of fractions 7–9 and 11. All the active fractions (fractions 7–15) contained *Z*-ligustilide, in which fractions 7–9 comprised the most abundant *Z*-ligustilide. Only a small sigmal for *Z*-ligustilide was detected in fraction 11. BP appeared to be present primarily in fractions 8 and 9.

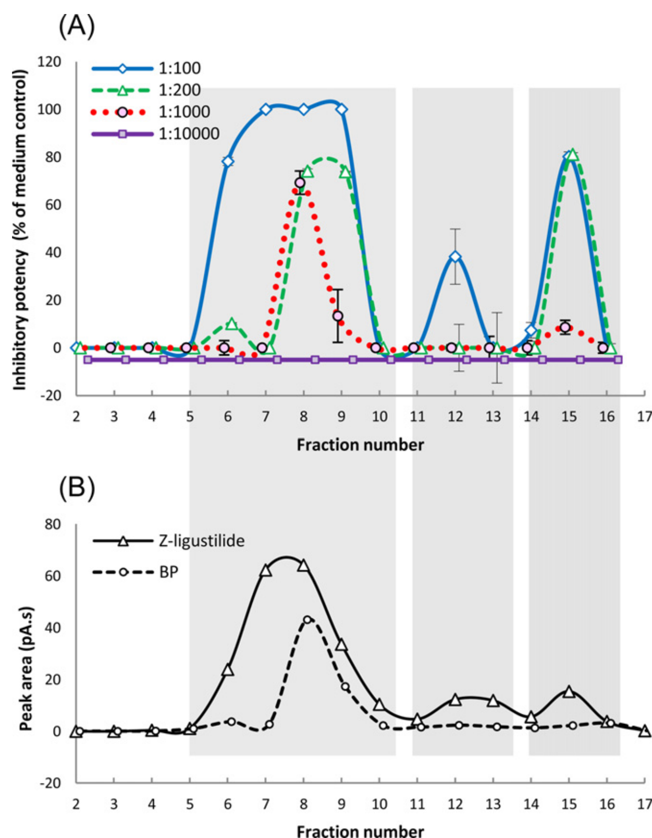

Fig. 6. Relationship between the bioactivity and the amount of Z-ligustilide and BP in the fractions. (A) Cultured endothelial cells were treated with a serial dilution (1:100, 1:200, 1:1000 and 1:10,000) of VOAS fractions. The bioactivity was determined by the MTS assay. The data of inhibitory potency are according to the reduction rate of endothelial cell viability and shown as percentage of medium control. (B) The Z-ligustilide and BP contents in the fractions were analyzed and measured by GC. The GC results are expressed as peak area (pA.s). There is a strong correlation shown between the bioactivity and the presence of Z-ligustilide and BP in the fractions.

Z-ligustilide and BP present in the fractions. For example, fraction 8 which contained the highest amount of *Z*-ligustilide and BP among all fractions was the most potent fraction, with its 1:1000 dilution remaining highly active. In contrast, fraction 14 was much less active and contained relatively small amount of *Z*-ligustilide and BP.

In the current study, we achieved partial separation of *Z*-ligustilide from BP in 30 min (2 min/fraction) (Fig. 6B) In contrast, the protocol by Zhang et al. [16] took 500 min (8.5 h). They used phase system *n* hexane–ethyl acetate–methanol–water–acetonitrile in the ratio of 8:2:5:5:3 (v/v) to purify *Z*-ligustilide in two steps. First step was supercritical fluid extraction from the raw herb, the dried roots of *Ligusticum chuanxiong*, and the second step used high-speed CCC for the separation and purification of the compound. Moreover, Wang et al. [4] used two-phase system petroleum ether (60–90 °C)–ethanol–water in the ratio of 10:17:10 (v/v) to purify *Z*-ligustilide by high-speed CCC. Their CCC run took 3 h.

Moreover, Fig. 6A shows that although the bulk of the *Z*-ligustilide eluted as one major peak, the two minor peaks eluted later were also associated with bioactivity. This suggests the possibility of *Z*-ligustilide eluting in different forms, either physically or in association with other molecules.

3.4. Synergistic action of the active substances in VOAS

Collectively, our results provide strong evidence that *Z*-ligustilide and BP were the main composite compounds responsible for the anti-endothelial properties of VOAS. To confirm this, we examined the effects of *Z*-ligustilide and BP alone on endothelial cells and compared with VOAS. Fig. 7 shows that *Z*-ligustilide and BP decreased HUVEC viability in a concentration-dependent manner, and the significant inhibitory effects were observed both at concentration range 40–100 μg/mL ($P < 0.05$, unpaired Student's *t*-test). Nevertheless, VOAS appeared to produce a much stronger effect than *Z*-ligustilide or BP alone: VOAS at the concentrations above 30 μg/mL caused complete cell death. These results suggest the possibility that the activity of *Z*-ligustilide and BP are greatly enhanced by the presence of other unidentified components in VOAS through synergistic behavior, although these components were not detectable by GC.

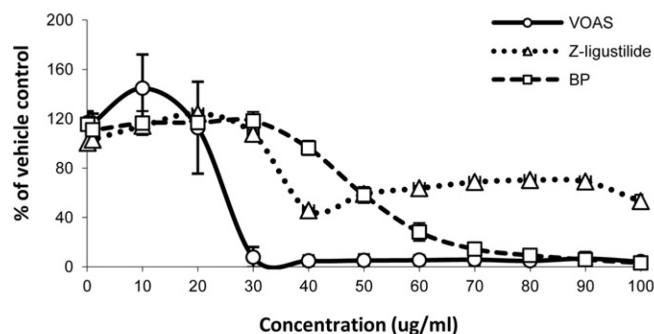

Fig. 7. Anti-endothelial properties of VOAS, Z-ligustilide and BP. HUVECs were incubated with VOAS, Z-ligustilide and BP at concentration range 0.1–100 μg/mL for 48 h. Cell viability was determined by adding 20 μl/well MTS reagent and the absorbance value at 492 nm were recorded. Data are expressed as % of vehicle control and are mean ± SD. Z-ligustilide and BP concentration-dependently reduced HUVEC viability and the significant inhibitory effects were observed both at concentration range 40–100 μg/mL ($P < 0.05$, unpaired Student's t-test). However, VOAS appeared to produce a much stronger effect than Z-ligustilide or BP alone, with concentrations higher than 30 μg/mL resulted in a complete cell death.

Similar findings were reported by Kan et al. [17] who showed a synergistic interaction of three phthalides, *Z*-ligustilide, BP and senkyunolide A. They found that a crude oil extract of *A. sinensis radix* produced a significantly stronger anti-proliferative activity on colon cancer HT-29 cells than a mixture of these three phthalides. The composition ratio of these three phthalides in the mixture was identical to that in the crude oil extract. Another possibility was that VOAS may somehow protect or preserve the bioactivity of *Z*-ligustilide and BP in the cell culture environment (37 °C, 5% CO_2 for 48 h). Indeed, *Z*-ligustilide is known for its instability and rapid degradation [18,19].

4. Conclusion

A frequently used procedure to demonstrate the chemical basis for bioactivities in an extract is to analyze the extract by a method such as HPLC, identify the compounds and then show they possess bioactivity. We describe another approach: the fractionation of the bioactivities in a crude extract by countercurrent chromatography followed by analysis of the chemical species present in the bioactive fraction. By this CCC procedure, coupled with a MTS cell viability assay and GC analysis, we have shown that *Z*-ligustilide and BP are the two main constituents that contribute to the anti-endothelial properties of VOAS. The fractionation was achieved without the loss of the original activity and is thus a rapid and reliable approach to identify potential active compound in crude plant extract. The CCC method also offers the possibility of isolating purified active species. This approach can be particularly useful for natural products with multiple active species and substances with synergistic interactions as the method is led by the bioactivity rather than the separated compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.03.013.

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