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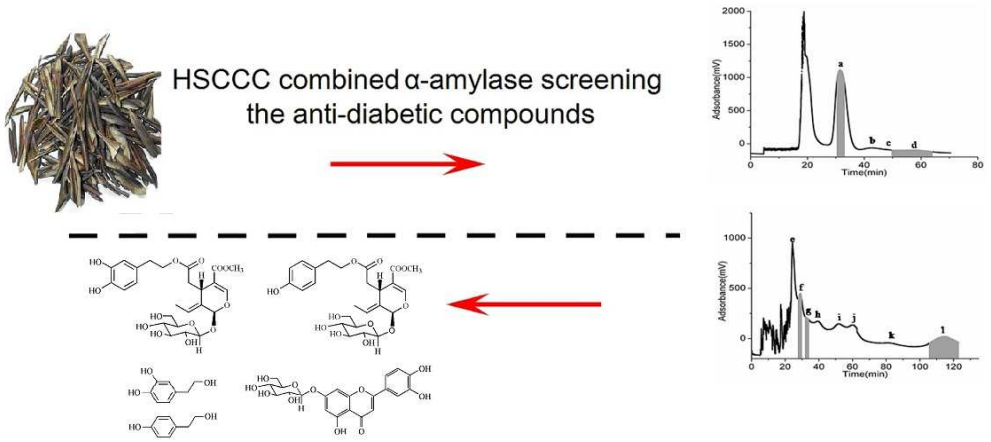

This paper proposed a novel strategy of screening anti-diabetic compounds by HSCCC coupled with post-column on-line α-amylase evaluation.

Efficient method for screening and identification of anti-diabetic components in the leaves of *Olea europaea* L.

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Abstract

In this article, an efficient method, high speed counter-current chromatography (HSCCC) coupled with post-column on-line evaluation was developed to screen, isolate and identify the major anti-diabetic compounds in the leaves of *Olea europaea* L. The HSCCC separation employed a two-step process: first, an optimized two-phase system that composed of ethyl acetate-water (1:1) was used to separate the extraction; then, a solvent system that composed of butanol-water-acetic acid (1:1:0.1) was applied to the further separation of the anti-diabetic active compounds. The eluant was detected by post-column evaluation with α-amylase on both steps. It was found

that five major constituents in the extract of the leaves of *O. europaea* L possessed potential anti-diabetic activities. Their structures were identified as Oleuropein, Ligstroside, Hydroxytyrosol, Tyrosol and Luteolin-7-O-*β*-D-glucoside by ^{1}H- and ^{13}C-Nuclear Magnetic Resonance (NMR).

Keywords Leaves of *Olea europaea* L, Anti-diabete, High speed counter-current chromatography, On-line activity screening

1 Introduction

The Olive tree (*Olea europaea* L.) is one of the very important fruit trees in Mediterranean countries. Epidemiological studies have shown a relationship between the Mediterranean diet and a lowered incidence of pathologies such as cardiovascular diseases, cancer and diabetes[1]. Several studies attribute these health benefits to high consumption of virgin olive oil which is rich in phenols and flavonoids as well as other typical components of the Mediterranean diet[2]. Many data on the polyphenols of olive fruits[3] and olive oil[4] have been reported, but a few studies have been published on olive leaves[5, 6]. In fact, olive leaves which are considered as byproduct of olive trees are the best source of oleuropein and related compounds. Those compounds have been shown to have multifunctional bioactive properties related to radical scavenging activity[7, 8]. Meanwhile, olive leaves were used to treat and prevent hypertension in popular medicine and phytotherapy. And there has report that the leaves also have the activity of anti-diabeties, but the active ingredients are often difficult to separate and purify directly from crude extracts due to their high polarity.

Diabetes mellitus is one of the global major diseases. The World Health Organization (WHO) estimated that there are 346 million people worldwide who suffer from diabetes, and this figure will double by the year 2030. Diabetes is a group of metabolic diseases but the predicted increase is mainly in type Ⅱ diabetes[9]. Type Ⅱ diabetes mellitus, by far the most common type, is a metabolic disorder of multiple etiology characterized by carbohydrate, lipid and protein metabolic disorders. And it includes defects in insulin secretion, almost always with a major contribution of insulin resistance[10]. These abnormalities could lead to many lesions, such as retinopathy, nephropathy, neuropathy and angiopathy[11]. Consequently, the inhibition of carbohydrate digestive enzymes is considered a therapeutic tool for the treatment of type Ⅱ diabetes[12]. The most important digestive enzyme is pancreatic α-amylase, a calcium metalloenzyme that catalyzes the hydrolysis of the α-1, 4 glycosidic linkages of starch, amylose, amylopectin, glycogen and various maltodextrins. Furthermore, it is responsible of most of starch digestion in humans[13]. Thus, the inhibition of α-amylase activity is considered as an effective strategy to control diabetes because the inhibitors of α-amylase are effective in delaying glucose absorption by means of breaking down long-chain carbohydrates. From this point of view, more researchers have focused on the search for more effective inhibitors of α-amylase from natural materials for anti-diabetes[14, 15], such as polysaccharides from tea leaves[16], hydrolysate from sardine muscle and isoflavones from soybean[17].

It is a time-consuming and hard work to screen the natural hypoglycemic agents from complex mixtures. HSCCC is now accepted as one of the common techniques

for the separation and purification of various compounds from natural products[18]. HSCCC is a liquid chromatography working without a solid stationary phase and it has the ability of large-scale purification[19]. As we know, in order to obtain a suitable two-phase system for HSCCC, the polarities of the solvents chosen should be differ significantly and n-butanol/water is one of the most polar solvent systems for the separation.

Thus, in this study, different fractions Ⅰ-Ⅵ were obtained by reflux extraction and separated with macroporous resins from the leaves of *Olea europaea* L. Then, the α-amylase system was used to evaluate the inhibitive effects for anti-diabetic activities of the 6 fractions. The results showed that the fraction Ⅳ has a better anti-diabetic effect than other fractions. We proposed a novel strategy of HSCCC purification for the efficient and effective discovery of anti-diabetic compounds from fraction Ⅳ based on a post-column on-line method with anti-diabetic assay by α-amylase. The separation contained two steps: at first, an optimized two-phase system that composed of ethyl acetate–water (1:1) was used to separate the extraction; then, a solvent system composed of butanol–water–acetic acid (1:1:0.1) was applied to the further separation of the anti-diabetes active components. The eluant was evaluated by post-column on-line evaluation with α-amylase anti-diabetic assay on both steps. We found that there have five major constituents in the extract of the leaves of *O. europaea* L possessed potential anti-diabetes activities. Their structures were identified as Oleuropein, Ligstroside, Hydroxytyrosol, Tyrosol, Luteolin-7-O-*β*-D-glucoside by ^{1}H-NMR and ^{13}C-NMR (Fig.1). In this way, a method

which is combination of liquid-liquid partition chromatography and α-amylase on-line evaluation was accomplished to screening the anti-diabetic compounds. And the method would make it possible to use HSCCC for the preparative isolation and screening of the anti-diabetic high polarity compounds from *Olea europaea* L.

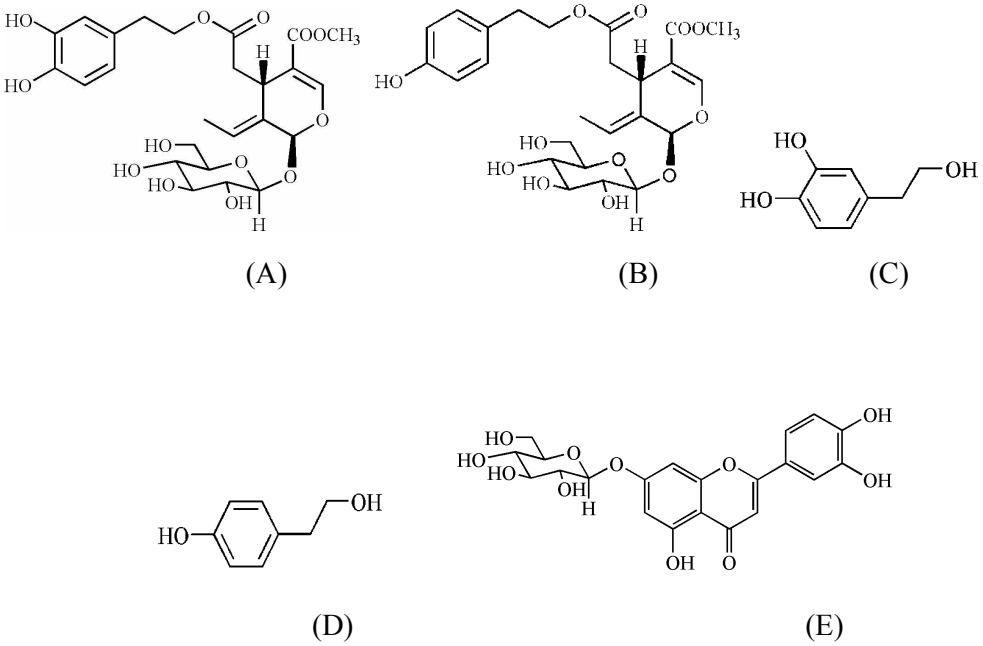

Fig.1 Structures of the anti-diabetic compounds: (A) Oleuropein, (B) Ligstroside, (C) Hydroxytyrosol, (D) Tyrosol, (E) Luteolin-7-O-*β*-D-glucoside.

2 Experimental

2.1 Apparatus

In the present study, a Spectrum HSCCC instrument (DE Spectrum Centrifuge) (Dynamic Extractions Co. Ltd., Slough, UK) equipped with two bobbins was employed. Each bobbin of the apparatus fits one analytical column and one preparative column made by polytetrafluroethylene (PTFE). The column volume of

each analytical column and preparative column is 14.0 mL of 0.8 mm ID and 72.0 mL of 1.6 mm ID, respectively. The β-value is defined as $\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge. In this case, β varied from 0.64 at the internal terminal to 0.81 at the external terminal for the analytical columns, and it is 0.52 to 0.86 for preparative columns. The maximum rotational speed of the instrument is limited to 1600 rpm. A rotation rate of 1400 rpm was used in this study giving a high "*g*" value of 240 × *g*. The HSCCC separation setup consisted of two preparative pumps H&E P3000 A, and a spectrophotometer H&E UV3000 (H&E Co. Ltd., Beijing, China) with a preparative cell operating at 280 nm. The separation temperature was controlled by a DLSB-10/40ºC constant temperature regulator (Yarong Instruments Co. Ltd., Zhengzhou, China). A CBS-A automatic fraction collector (Shanghai Huxi Analysis Instrument Factory Co. Ltd., Shanghai, China) was used to collect the fraction.

The HPLC analysis was performed in an Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA) LC system, which was equipped with a G1322A vacuum degasser, a G1311 A quaternary pump, a G1315B diode array detector (DAD) performing the wavelength scanning from 190 to 950 nm and a G1328B manual injection valve. The system was controlled by Agilent Chemstation software (version A.10.02) (Agilent Technologies, Palo Alto, CA, USA). The separation was achieved on a Sinochrom ODS-AP C_{18} analytical column (Dalian Elite Analytical Instruments Co., Dalian, China, 250×4.6 mm i.d., 5 μm).

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Varian Inova-400 FT-NMR spectrometer (400 and 100 MHz, respectively).

2.2 Reagents and Samples

α-amylase from Bacillus licheniformis, 4-nitrophenyl a-D-glucopyranoside, dimethyl sulfoxide and starch were purchased from Shanghai Baoman Biological Technology Co. (Shanghai, China). Analytical-grade methanol, acetic acid, ethyl acetate and butanol were purchased from Tianjin Chemical Reagent Corporation (Tianjin, China). Deionized water and the solvents used for the activity screening method were filtered and degassed by passing through a 0.45 μm membrane filter before use. All organic solvents used for extraction and the HSCCC seperation were of analytical grade and glass-distilled prior to use.

The leaves of *O. europaea* L. were collected in the city of Longnan, Gansu Province, China. The plant was identified by Professor Huanyang Qi in Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, China and a voucher specimen was deposited at Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, China.

Macroporous resin AB-8 was purchased from Sunresin Technology Co. Ltd (Xi'an, China); the resin was pretreated by soaking in ethanol for 24 h, and then washed with ethanol until there was no turbidity when a threefold volume of water was added into the eluent. The resin was subsequently washed with distilled water until the ethanol was thoroughly replaced by the distilled water before use.

152

153 **2.3 Sample preparation**

154 The dried sample (approximately 250 g) was refluxed with 2.5 L 60% ethanol for 1 h
155 and repeated twice. The combined extracts were evaporated by a rotary evaporator in
156 vacuo at temperature not higher than 50℃. Then the residue was dissolved in a 1000
157 mL volumetric flask with water. The total extraction was named fraction Ⅰ.

158 Then this solution was divided into five parts, each 150 mL, then subjected to the
159 adsorption procedure for five columns in parallel, which was carried out in a glass
160 column (20 cm×2 cm i.d.) packed with macroporous resin (AB-8). The first column
161 was washed with distilled water of 6 BV at a flow rate of 3 BV/h. The obtained eluant
162 was evaporated to dryness under reduced pressure, named fraction Ⅱ. The other
163 columns were first washed with distilled water of 6 BV at a flow rate of 3 BV/h and
164 then eluted with aqueous methanol in different ratios of 6 BV at a flow rate of 3 BV/h.
165 The eluants were evaporated to dryness under reduced pressure, named fraction Ⅲ,
166 Ⅳ, Ⅴ and Ⅵ, respectively.

167

168 **2.4 Activity analysis for anti-diabetes**

169 It was using α-amylase to screen the anti-diabetes activities fraction in the vitro. The
170 α-amylase inhibitory assay was performed according to the method previously
171 described with slight modification[20, 21]. The total assay mixture composed of 0.1 mL
172 of a-amylase solution (1 unit/mL, distilled water), 0.15 mL of sample solutions at
173 different concentrations (in 5% DMSO). Following incubation at 37 ℃ for 10 min

and then 0.4 mL of soluble starch (0.4% *w*/*v*) in 25 mmol/L sodium phosphate buffer (pH7.0) was added to start the reaction. The reaction was carried out at 37℃ for 10 min and terminated by addition of 0.3 mL of HCl solution (1.0 mol/L). Next added 0.2 mL Iodine solution to the reaction system so that the system has color and diluted reaction liquid volume to 4 mL. The absorbance was measured at 660 nm by UV detection. For all tests, the inhibition assay was performed in triplicate. Inhibition activity of α-amylase was determined by a water-containing control which compared with and calculated by the following equation:

$$\alpha\text{-amylase activity inhibition rate} = \frac{(A_A - A_B) - (A_C - A_D)}{(A_A - A_B)} \times 100\%$$

where A_A was the optical density of reaction blank, the reaction blank mixture contained the same volume of the buffer solution instead of the sample; A_B was the optical density of the reaction in the presence without α-amylase; A_C was the optical density of the reaction in the presence of both a-amylase and olive sample; A_D was the optical density of the reaction in the presence of olive sample.

2.5 Selection of the two-phase solvent system

Successful separation by HSCCC depends on the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (*K*) for target compounds. Measurement of *K* values from crude sample was as follows: crude sample (20 mg) was weighed into a 10 mL test tube and added 5 mL of each phase of a pre-equilibrated two-phase solvent system. The test tube was then shaken vigorously for 10 min to thoroughly equilibrate the sample between the two phases. After settling,

1 mL of each phase was transferred to two separate test tubes and evaporated to dryness. The residues were diluted with 1 mL methanol and analyzed by HPLC. The *K* value was expressed as the peak area of target compounds in the upper phase (stationary phase) divided by that in the lower phase (mobile phase).

2.6 Preparation of two-phase solvent system and sample solution

In this study, the separation contained two steps. In the first step, the selected two-phase solvent system was composed of ethyl acetate–water with the volume ratio of 1:1 (*v*/*v*). In the second step, the selected two-phase solvent system was composed of butanol–water–acetic acid with the volume ratio of 1:1:0.1 (*v*/*v*/*v*). The solvent mixture was vigorously shaken several times and equilibrated in a separation funnel at room temperature. Then the upper phase and the lower phase were separated and degassed by sonication for 30 min prior to use.

The sample solution was prepared by dissolving 120 mg crude sample in 6 mL mixture solution of upper phase and lower phase (1:1 *v*/*v*) used for HSCCC separation.

2.7 HSCCC coupled with α-amylase on-line separation

According to the α-amylase activity result, selected fraction Ⅳ had better anti-diabetic activity. Thus, we screened the specific anti-diabetic compounds based on on-line HSCCC method with α-amylase assay. This method was created by adding the α-amylase in the fraction collection tube for the rapid screening of the

anti-diabetic compounds in a complex mixture. The separation was carried out on preparative column (142 mL) and contained two steps. In each separation run, the multiple layer coiled column was first entirely filled with the upper phase (stationary phase) of ethyl acetate–water (1:1 *v*/*v*) in the head to tail mode. Then the lower phase (mobile phase) of ethyl acetate–water (1:1 *v*/*v*) was pumped into the column at a flow rate of 4.8 mL/min while the rotor was rotated at 1400 rpm. When hydrodynamic equilibrium was established, 6 mL of the sample solution (20 mg/mL) was injected into the column through the sample valve. In the first step, the mobile phase of ethyl acetate–water (1:1 *v*/*v*) was used and the flow rate was 4.8 mL/min. Through the run, the separation temperature was controlled at 30°C. The effluent from the tail end of the column was continuously monitored at 280 nm with the UV detector. Fraction collection was started at the time of injection. In total, fractions were collected in 2 min intervals. These fractions were combined according to their composition and evaporated to dryness in the SpeedVac. Each fraction was evaluated by above method with α-amylase activity method.

As the fraction 1 obtained from the first step was admixture, further separation was performed for the second step. Fraction 1 was collected and evaporated under reduced pressure to dryness. The second step was filled with the upper phase (stationary phase) of butanol–water–acetic acid (1:1:0.1 *v*/*v*/*v*) in the head to tail mode. Then the lower phase (mobile phase) of butanol–water–acetic acid (1:1:0.1 *v*/*v*/*v*) was pumped into the column at a flow rate of 3 mL/min while the rotor was rotated at 1500 rpm. When hydrodynamic equilibrium was established, 1 mL of the fraction 1 sample solution

(20 mg/mL) was injected into the column through the sample valve. Through the run, the separation temperature was controlled at 20 °C. The effluent from the tail end of the column was continuously monitored at 280 nm with the UV detector and fraction collection was started at the time of injection with 2 min intervals. These fractions were combined according to their composition and evaporate to dryness in the SpeedVac. Each fraction was evaluated by the above method with α-amylase activity method.

2.8 HPLC analysis and identification of HSCCC peak fractions

The crude sample and fractions separated by HSCCC were all analyzed by HPLC. The analysis was achieved with SinoChrom ODS-AP C_{18} analytical column at a temperature of 30°C. The mobile phase consisted of solvent A (water) and B (methanol). A liner gradient elution program was used as follows: 0-20 min, 1%-100% B. The flow rate was kept at 1.0 mL/min and the detection wavelength was set at 280 nm. The injection volume was 20 μL each time and all of the mobile phase was filtered through a 0.45 μm Millipore filter before use.

2.9 Identification of anti-diabete compounds with NMR

A semi-preparative HPLC system was used to isolate the constituents from active fractions. The semi-preparative chromatographic conditions applied to the separations were based on the elution profile observed during the HPLC separation. They were optimized for baseline separation of the compounds. The yield per compound was

typically in the milligram range. The ^{1}H-NMR and ^{13}C-NMR spectral of compounds A-E were recorded on a Varian Inova-400 FT-NMR spectrometer (USA) with DMSO-d_6 as the solvent. Chemical shifts were given on a δ (ppm) scale with tetramethylsilane as the internal standard. These compounds were selected as they are the major anti-diabetic active constituents of olive leaves. Date of each compound given as follows:

Oleuropein (A): a yellow gum, ^{1}H-NMR (400 MHz, DMSO-d_6): δ 5.86 (s, 1H, H-1), 7.50 (s, 1H, H-3), 3.85 (m, 1H, H-5), 2.39 (m, 1H, H-6), 5.94 (m, 1H, H-8), 1.63 (d, 3H, J = 6.0 Hz, H-10), 3.66 (s, 3H, OMe), 4.05 (m, 2H, H-1′), 2.67 (m, 2H, H-2′), 6.58 (s, 1H, H-4′), 6.62 (d, J = 8.0 Hz, 1H, H-7′), 6.46 (d, J = 8.0 Hz, 1H, H-8′), 4.63 (d, J = 8.0 Hz, 1H, glc-1"); ^{13}C-NMR (100 MHz, DMSO-d6): δ 92.9 (C-1), 153.3 (C-3), 107.7 (C-4), 30.1 (C-5), 39.9 (C-6), 170.7 (C-7), 123.1 (C-8), 128.4 (C-9), 13.0 (C-10), 166.2 (C-11), 51.3 (OMe), 65.1 (C-1′), 33.7 (C-2′), 129.1 (C-3′), 116.2 (C-4′), 145.1 (C-5′), 143.8 (C-6′), 115.5 (C-7′), 119.6 (C-8′), 99.0 (glc-1"), 73.3 (glc-2"), 76.5 (glc-3"), 69.9 (glc-4"), 77.4 (glc-5"), 61.1 (glc-6"). Those data were in agreement with earlier published data for Oleuropein[22].

Ligstroside (B): a yellow gum, ^{1}H-NMR (400 MHz, DMSO-d_6): δ 5.91 (s, 1H, H-1), 7.47 (s, 1H, H-3), 3.86 (m, 1H, H-5), 2.39 (m, 1H, H-6), 6.01 (m, 1H, H-8), 1.63 (d, 3H, J = 6.0 Hz, H-10), 3.66 (s, 3H, OMe), 4.19 (m, 2H, H-1′), 2.78 (m, 2H, H-2′), 7.05 (d, 3H, J = 8.8 Hz, H-4′), 6.76 (d, 3H, J = 8.0 Hz, H-5′),6.76 (d, 1H, J = 8.0 Hz, H-7′), 7.05 (d, 1H, J = 8.8 Hz, H-8′), 4.84 (d, 1H, J = 8.0 Hz, glc-1"); ^{13}C-NMR (100 MHz, DMSO-d6): δ 92.9 (C-1), 152.8 (C-3), 107.3 (C-4), 29.8 (C-5), 33.8 (C-6),

170.4 (C-7), 122.6 (C-8), 127.7 (C-9), 11.9 (C-10), 165.7 (C-11), 50.0 (OMe), 64.6 (C-1′), 33.0 (C-2′), 128.5 (C-3′), 129.1 (C-4′), 114.4 (C-5′), 155.2 (C-6′), 114.4 (C-7′), 129.1 (C-8′), 98.7 (glc-1"), 72.7 (glc-2"), 75.8 (glc-3"), 69.5 (glc-4"), 76.1 (glc-5"), 61.0 (glc-6"). Those data were in agreement with earlier published data for Ligstroside[23].

Hydroxytyrosol (C): a yellow gum, ^{1}H-NMR (CD_3COCD_3): δ 6.65 (m, 2H, H-2, 5), 6.51(d, 1H, *J* =8.0, 2.0 Hz, H-6), 2.64 (t, 2H, *J* = 7.2 Hz, H-7), 3.65 (t, 2H, *J* = 7.6 Hz, H-8); ^{13}C-NMR (CD_3COCD_3): δ 130.0 (C, C-1), 114.3 (CH, C-2), 144.0 (C, C-3), 142.5 (C, C-4), 115.2 (CH, C-5), 119.3 (CH, C-6), 37.9 (CH_2, C-7), 62.6 (CH_2, C-8) . Those data were in agreement with earlier published data for Hydroxytyrosol[24].

Tyrosol (D): a yellow gum, ^{1}H-NMR (CD_3COCD_3): δ 6.71 (d, 2H, *J* =6.4, 2.4 Hz, H-3, 5), 7.01 (d, 2H, *J* = 6.4,2.4 Hz, H-2, 6), 3.63 (t, 2H, *J* = 7.2 Hz, H-8), 2.67 (t, 2H, *J* = 7.2 Hz, H-7); ^{13}C-NMR (CD_3COCD_3): δ 155.6 (C, C-4), 114.9(CH, C-3,5), 129.8(CH, C-2,6), 129.7(C, C-1), 63.2 (CH_2, C-8), 38.5 (CH_2, C-7) . Those data were in agreement with earlier published data for Tyrosol[24].

Luteolin-7-O-*β*-D-glucoside (E): a yellow powder, ^{1}H-NMR (DMSO-d_6): δ 7.95 (d, 2H, *J* = 8.8 Hz, H-2', 6'), 6.92 (d, 2H, *J* = 8.8 Hz, H-3', 5'), 6.87 (s, 1H, H-3), 6.82 (d, 1H, *J* = 2.0Hz, H-8), 6.43 (d, 1H, *J* = 2.0Hz, H-6), 3.15-5.43 (H-Glc); ^{13}C-NMR (DMSO-d_6): δ 164.3 (C-2), 103.2 (C-3) , 181.9 (C-4), 161.1 (C-5), 99.5 (C-6) , 162.9 (C-7), 94.7 (C-8) , 156.9 (C-9) , 105.3 (C-10) , 121.4 (C-1′), 113.5 (C-2′) , 145.8 (C-3′) , 149.9 (C-4′) , 116.0 (C-5′) , 119.2 (C-6′), 99.7 (glu-1) , 73.1 (glu-2) , 76.4 (glu-3), 69.5 (glu-4) , 77.1 (glu-5) , 60.6 (glu-6)。 Those data were in agreement with

306 earlier published data for Luteolin-7-O-β-D-glucoside[25].

307 And the compounds A-E were further analyzed on HPLC to determine their purities.
308 The purities of Oleuropein, Ligstroside, Hydroxytyrosol, Tyrosol and
309 Luteolin-7-O-β-D-glucoside elution peaks were 91, 93, 87, 89 and 82%, respectively
310 assessed by HPLC at 280 nm.

311 **3 Results and Discussion**

312 **3.1 Anti-diabetic activity**

313 The inhibition activity of carbohydrate hydrolyzing enzymes in the human digestive
314 tract is regarded as an effective method for the control of diabetes by diminishing the
315 absorption of glucose decomposed from starch by these enzymes. Therefore, effective
316 and nontoxic inhibitors of α-amylase have long been sought. The purified six fractions
317 from the olive leaves by resin AB-8 were screened for their anti-diabetic activities by
318 the α-amylase assay, which is widely used to evaluate the anti-diabetic activities of
319 natural products. The result is summarized in Tab. 1. IC_{50} values of the Ⅰ, Ⅲ, Ⅳ,
320 Ⅴ and Ⅵ against α-amylase was 249.0, 49.3, 34.1, 55.0 and 39.3 mg/mL,
321 respectively. The fraction Ⅱ showed relative weak anti-diabetic activity with IC_{50}>
322 250 mg/mL. The results suggested that water/ethanol elution parts displayed the
323 anti-diabetic activity against α-amylase and fraction Ⅳ showed stronger inhibitory
324 activity than others.

325 Tab. 1 Results of inhibitory rates on α-amylase of different fractions ($\bar{x}$ ±s, n=3)

Inhibition rate （%） \ Concentration （mg/mL）	30	40	50	60	70	80	90	100

Fraction Ⅰ	26.1±1.4	26.5±0.8	28.6±1.1	29.5±1.3	31.8±0.5	36.6±2.1	38.1±1.8	38.8±1.6
Fraction Ⅱ	5.6±0.3	6.3±0.2	7.3±0.4	6.8±0.3	5.9±0.3	7.5±0.4	8.1±0.3	8.1±0.3
Fraction Ⅲ,	27.5±0.8	36.4±0.6	48.0±2.6	54.5±2.3	66.7±2.8	75.6±4.3	85.6±1.3	87.7±2.6
Fraction Ⅳ	57.5±0.3	60.0±0.8	65.5±2.1	70.0±0.8	74.4±1.0	85.2±1.2	93.9±1.8	96.9±2.8
Fraction Ⅴ	19.5±1.0	35.8±1.1	45.9±0.5	55.3±1.3	60.9±1.1	68.7±3.6	72.9±2.2	80.9±1.8
Fraction Ⅵ	42.6±0.7	54.4±1.0	59.7±1.0	65.1±1.2	72.7±1.4	79.6±0.9	93.2±0.3	94.7±1.2

3.2 Optimization of HSCCC conditions for separation of the anti-diabetic fraction

The optimum of the separation conditions for HSCCC is related to various parameters including a two-phase solvent system, flow rate, revolution speed, column temperature, et al. An appropriate solvent system providing a suitable range of the partition coefficients (K) for target compounds is the key to a successful HSCCC separation. As previously reported, for effective separations with regard to resolution and short elution time, partition coefficient (K) at the range of $0.5<K<2$ is required. If the K value is too low, the target peak won't properly separate from other peaks; if the K value is too high, it will lead to long run times and the consumption of excessive solvent. In order to obtain the optimal two-phase solvent system for the separation, the HEMWat solvent was chosen to be tested. HEMWat is a classic two-phase solvent system because it provides a broad polarity range[26]. In this experiment, the K values of the target compounds in different ratios of HEMWat were determined by HPLC and listed in Table 2. The large differences of K values in different HEMWat solvent

systems showed that the target compounds covered a wide polarity range, indicating that it was impossible to separate them with a conventional HSCCC method. Thus, we developed two steps to separate the target compounds with HSCCC. In order to select the optimum two-phase solvent system, a series of tests were performed in this study. According to the rules of selecting the optimal conditions, in the first step, several types of the solvent systems composed of ethyl acetate–n-butanol–water at different volume ratios were chosen. The measured *K* values were summarized in Tab 2. It was found that ethyl acetate–water (1:1 *v*/*v*) solvent system provided suitable *K* values for the target compounds because of their *K* values being in the range 0.5-2 allowing efficient separation within a comfortable running time.

As it is difficult to isolate all polar compounds from the extraction, we chose the fraction 1 which was admixture obtained from the first step for further separation in the second step. In order to obtained significant differences in the *K* values, in this step, the same measured method for *K* values was used, and butanol–water–acetic acid (1:1:0.1 *v*/*v*/*v*) was used as the *K* values was obvious and helpful for obtaining good separation of the compounds (Tab. 3).

Tab. 2 The *K* values of HSCCC solvent system in the first step

NO.	Extraction solvent	*K* values			
		K_a	K_b	K_c	K_d
1	Heotane–EtOAc–MeOH–Water (1:6:1:6)	0.1	0.23	0.40	0.29
2	Heotane–EtOAc–MeOH–Water (1:9:1:9)	0.36	0.37	0.67	1.7
3	Heotane–EtOAc–MeOH–Water (1:19:1:19)	0.41	0.53	1.24	0.36

4	EtOAc–Water (1:1)	0.66	0.74	1.45	0.52
5	EtOAc–MeOH–Water (4:1:5)	10.6	>10	>10	>10

359

360 Tab. 3 The *K* values of HSCCC solvent system in the second step

NO.	Extraction solvent	*K* values							
		K_e	K_f	K_g	K_h	K_i	K_j	K_k	K_l
1	EtOAc–Butanol–water (2:3:5)	0.69	0.41	0.12	0.89	8.05	4.28	2.02	4.27
2	Butanol–water (1:1)	0.36	1.13	0.31	0.59	5.20	3.08	1.64	3.97
3	Butanol–water–acetic acid (1:1:0.1)	0.47	1.72	0.22	0.60	0.58	1.43	0.76	0.68
4	Butanol–water–acetic acid (1:1:0.3)	0.53	0.81	0.49	0.62	1.59	1.99	0.90	2.05

361 The high flow rate of the mobile phase shortened the separation time, while the low
362 flow rate phase improved the resolution. HSCCC was carried out by using the upper
363 layer of solvent as the stationary phase and the lower layer of solvent as the mobile
364 phase. Based on the preliminary experiment, the flow rate and rotation speed were
365 optimized, where a flow rate of 4.8 mL/min combined with a rotation speed of 1400
366 rpm in the first step resulted in a good separation of sample within a decent time
367 period (200 min). In the second step, the separation was achieved at a flow rate of 3.0
368 mL/min combined with a rotation speed of 1500 rpm and resulted in a good
369 separation of sample within a decent time period (100 min). Under the optimized
370 conditions, the compounds were separated from the sample solution with the

concentration 20 mg/mL.

3.3 Evaluation of specific antioxidants in natural products by post-column on-line HSCCC with α-amylase anti-diabetic assay

Recently, on-line HPLC with DPPH radical scavenging assay has been focused on the screening of specific antioxidants from various samples such as espresso coffees, olive leaf, and non-polar food matrixes[27]. This method was useful to indicate unknown antioxidants from natural products and foods. However, on-line HPLC with DPPH radical scavenging assay has disadvantages that these targets of anti-oxidative activity could not be purified for the identification and/or evaluation of unknown compounds by NMR, *in vivo*, and *in vitro* assays. Thus, in order to discover the anti-diabetic compounds, we developed a novel strategy using post-column on-line HSCCC with α-amylase anti-diabetic assay. It would be a valid and feasible way for screening unknown anti-diabetes from the leaves of *Olea europaea* L. Consequently, the first step of post-column on-line HSCCC with α-amylase anti-diabetic assay was adding 0.15 mL α-amylase to the each sample collection tubes behind column of HSCCC. Then the HSCCC separated the fraction Ⅳ with the optimal two-phase solvent system. The effluent from the tail end of the column was continuously

monitored at 280 nm with the UV detector and fraction collection was started at the time of injection. Fractions were collected according to their composition in the column with α-amylase. Following reaction for 10 min, 0.4 mL of soluble starch (0.4%, *w*/*v*) in 25 mmol/L sodium phosphate buffer (pH7.0) was added. It was carried out for 10 min and terminated by addition of 0.3 mL of HCl solution (1.0 mol/L), then add 0.2 mL Iodine solution to have color. The absorbance was measured at 660 nm by UV detection. The same method was used in the second process. The method showed that a very useful and efficient purification method for specific anti-diabetes from olive leaves was developed by HSCCC using the simple two-phase solvent system. These results were shown in Fig.2. We can see peak a and d (compound A, B) in the first step and peak f, g and l (compound C, D and E) showed the anti-diabetic activity.

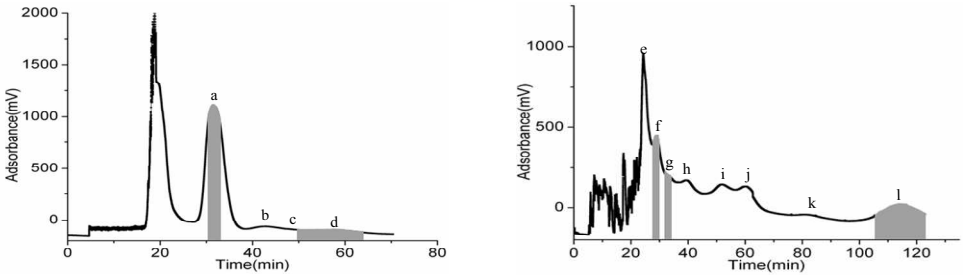

Fig. 2 HSCCC chromatogram of fraction Ⅳ HSCCC chromatogram of fraction 1

4 Conclusions

In this study, we successfully applied the novel high speed counter-current

chromatography (HSCCC) coupled with post-column on-line evaluation method to the rapidly and efficient screening five major anti-diabetic compounds from the leaves of *Olea europaea* L. with acceptable purity. The results of the study demonstrated that it is a powerful method for separation and it can reduce the time taken to obtain the target compounds because the separation would be achieved under the guidance of on-line evaluation with potential anti-diabetic activity. Meanwhile, this method provided an instructive pattern for screening and identifying active substance in herbal medicines.

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