Preparative separation of cacao bean procyanidins by high-speed counter-current chromatography

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Abstract

In this work, an efficient method for preparative separation of procyanidins from raw cacao bean extract by high-speed counter-current chromatography (HSCCC) was developed. Under the optimized solvent system of n-hexane-ethyl acetate-water (1:50:50, v/v/v) with a combination of head–tail and tail–head elution modes, various procyanidins fractions with different polymerization degrees were successfully separated. UPLC, QTOF-MS and \textsuperscript{1}H NMR analysis verified that these fractions contained monomer up to pentamer respectively. Dimeric procyanidin B2 (purity > 86\%) could be isolated by HSCCC in a single run. Other individual procyanidins in these fractions could be further isolated and purified by preparative HPLC. The developed HSCCC together with preparative HPLC techniques appeared to be a useful tool for large preparation of different procyanidins from cacao beans. Furthermore, by antioxidant activity assays, it was proved that both fractions and individual procyanidins possessed greater antioxidant activities compared to standard trolox. The antioxidant activities of procyanidins increase as the increase of their polymerization degree.

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1. Introduction

Procyanidins are a subclass of flavonoids and are composed of the flavan-3-ol monomers that naturally occur in plants and are known to exhibit many physiological activities, including antioxidant, cardioprotective, anti-inflammatory, and enzyme inhibitory effects [1,2]. Various studies have shown that health benefits of procyanidins are related to their structures [3,4], and the structural diversity is due to the type of interflavanoid linkage, the kind and number of flavan-3-ol units. Most oligomeric procyanidin (OPCs) and polymeric procyanidin (PPCs) are linked through C4 → C8 and/or C4 → C6 interflavan bonds as (+)-catechin and/or (−)-epicatechin as basic unit.

Procyanidins are found in various plant-derived foods, such as apple, pear, berries, wine, cacao, and nuts [5–9]. Cacao beans are the fruit of the \textit{Theobroma cacao} L., which is a plant originated in the rain forests of South and Central Americas. Though there are over 20 species in the genus, only \textit{Theobroma cacao} L. is widely cultivated [10–12]. Nowadays cacao and its associated products are consumed widely throughout the world.

Cacao beans are rich in polyphenols which account for about 12 to 18 in percent of the dry weight [13]. The presence of dimeric procyanidins B1 to B7, trimeric procyanidin C1, tetramer procyanidin (cinnamtannin A2) and pentameric procyanidin (cinnamtannin A3) in cocoa products has been reported [14,15]. A number of studies have shown that the content of procyanidins in cocoa products is higher than those in blueberry and cranberry on a dry weight basis and cocoa products has a higher antioxidant capacity than tea and red wine [16,17]. However, at present, there are few studies concerning raw cacao beans.

Procyanidins have been isolated from natural sources by extraction, fractionation and purification using traditional methods, such as...
as liquid-liquid extraction [18], microwave extraction [19], ultrasound extraction [20], thin-layer chromatography [21], column chromatography [18,22] and semi-preparative and preparative HPLC [23]. These methods are inevitably accompanied with some disadvantages. Sun et al. [18] used a combined solvent system for liquid-liquid extraction of stilbenes from grape skins. The established method permitted large preparation of crude polyphenols fractions, but very time- and solvent-consuming. Microwave extraction was used for preparation of polyphenols from apple pomaces [19], but the proposed method has disadvantages of low yield and high cost. The polyphenols were extracted from the unripe apple assisted using ultrasound [20], but accompanied with weakness of complex process and solvent-consuming. Thin-layer chromatography was applied for separation of flavan-3-ols from oak bark and green tea [21], however, this method was limited to the qualitative analysis and needed other techniques consociated for large scale preparation. Column chromatography permitted fractionation of proanthocyanidins from grape and wine [22], but complex process and very time-consuming lead it inefficiency. Semi-preparative HPLC was used for isolation of procyanidins from grape seeds [23], but complicated pretreatment and low yield were inevitable. Moreover, all of these methods presented disadvantage of secondary pollution because of the solid support matrix and/or repetitive sample injection. Compared to traditional techniques, in recent decades, a new chromatographic separation technique called high-speed counter-current chromatography (HSCCC) was developed, which is a support-free liquid-liquid partition chromatography based on hydrodynamic equilibration of the two-phase solvent system in the separation column. HSCCC technique provides several advantages such as higher partition efficiency in a shorter elution time [24–26], high sample recovery and large loading capacity. In previous work in our laboratory, by only one run of HSCCC for about 4 h, with a loading of 200 mg of monomer proanthocyanidins, (+)-catechin and (−)-epicatechin were succeeded in isolation as high as 78.9 mg and 52.5 mg, respectively. As compared, using traditional column chromatography, followed by semi-preparative HPLC, much less yields of polyphenols were obtained [23]. Another advantage of HSCCC technique over conventional column chromatography is no irreversible adsorption of sample onto the solid support [27]. Moreover, the high repeatability of HSCCC technique permits the established method in laboratory-scale applicable for large or industrial scale production. As a consequence, HSCCC technology has been widely used as an excellent method in the separation and purification of phenolic compounds from natural plants [24–26,28].

The aim of this work was to separate the proanthocyanidins in a preparative scale from raw cacao bean extract by an efficient method using HSCCC. Moreover, the antioxidant activity of the HSCCC fractions and individual procyanidins were verified, and the three common methods, i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP) assays, were used in this work.

2. Materials and methods

2.1. Standards and reagents

(+)-Catechin, (−)-epicatechin and procyanidin B2 were purchased from Chendu Must Bio-Technology Co., Ltd. (Chengdu, China). Chlorogluconol was purchased from Aladdin reagent (Shanghai, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), (±)-6-hydroxy-

2.5.7, 8-tetramethylchromane-2-carboxylic acid (trolox) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

All organic solvents used for HSCCC, UPLC and HPLC were purchased from Chemical Branch of Shandong Yuwang industrial Co., Ltd. (Shandong, China).

2.2. Materials

Freeze-dried cacao beans were provided by Polyphenol Laboratory of Pão Dois Portos/INIAV (Portugal). The preparation of the freeze-dried cacao beans was performed by using the fresh cacao purchased in a local market of Sao Tome and Principe, islands in the gulf of Guinea of the West African Coast. The cacao beans were isolated manually, washed with distilled water to eliminate residual pulp, frozen with liquid nitrogen, followed by lyophilization for 48 h.

2.3. Preparation of cacao bean phenolic extract

The cacao bean phenolic extract was prepared as report in our previous work [29]. Briefly, the desiccative raw cacao beans were finely ground using a high-speed miller (Tianjin TAISTE, type: FW 100). The powder was immediately used for the extraction of phenolic compounds and was defatted three times with n-hexane (solid/liquid 1:5, v/v), followed by sequential extraction using 80% aqueous methanol (v/v) and 75% aqueous acetone (v/v). The combined supernatants were evaporated at <30 °C to remove organic solvents, followed by three times extractions with an equal volume of chloroform to eliminate some alkaidal compounds. The aqueous phenolic solution was lyophilized and the powder obtained was stored at −20 °C until used.

2.4. HSCCC separation of cacao bean phenolic extract

2.4.1. Selection of solvent system

The partition coefficient (K) value and separation factor (α) were used as evaluation parameters for selection of the two-phase solvent system in HSCCC separation. In this work, the K values of cacao bean phenolic extract were determined by UV spectrophotometer based on reported method [27] with (−)-epicatechin, purified grape seed oligomeric procyanidins (OPCs), purified grape seed polymeric procyanidins (PPCs) as references. The K value was defined as $A_i / A_0$, where $A_i$ and $A_0$ meant the absorbance value of objective samples in lower stationary phase and the upper mobile phase, respectively. The separation factor (α) was the ratio of K values between two samples. Six solvent systems were selected (given in Table 1) and the K value and α value were obtained as follows. 0.5 mg of samples was added to 10 mL test tube with 3 mL of each phase of the above pre-equilibrated two-phase solvent system. The test tube was vigorously shaken and left to stand at room temperature until the equilibrium of the sample between the two phases was reached. 2 mL of each phase was evaporated to dryness and the residue was dissolved in 5 mL methanol to determine the absorbance at 280 nm.

2.4.2. Preparation of solvent system and sample solution

The optimized two-phase solvent system composed of n-hexane-ethyl acetate-water (1:50:50, v/v/v) was used for HSCCC separation of cacao bean phenolic extract. The solvent system in separatory funnel was shaken vigorously for mixing thoroughly and equilibrated at room temperature. The two phases were separated and degassed by ultrasonic device for 15 min. The lower aqueous phase was used as the stationary phase, and the upper organic phase as the mobile phase.

The sample solution was prepared by dissolving 400 mg of the freeze-dried cacao bean phenolic extract into 20 mL of lower phase...
Table 1
Partition coefficient values \((K)\) and separation factors \((\alpha)\) of \((-\)epicatechin, OPCs, PPCs and cacao bean extract.

<table>
<thead>
<tr>
<th>Solvent system ((v/v))</th>
<th>((-)Epicatechin ([1])</th>
<th>OPCs ([2])</th>
<th>PPCs ([3])</th>
<th>Cacao bean extract ([4])</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Butanol–EtOAc–H₂O 1–20–20</td>
<td>(K) 0.257</td>
<td>0.612 (2/1)</td>
<td>10.415</td>
<td>1.941</td>
</tr>
<tr>
<td>MeOH–EtOAc–H₂O 1–25–25</td>
<td>(K) 0.371</td>
<td>0.867 (2/1)</td>
<td>15.382</td>
<td>2.640</td>
</tr>
<tr>
<td>EtOAc–H₂O 1–50–10</td>
<td>(K) 0.407</td>
<td>1.028 (2/1)</td>
<td>16.159</td>
<td>2.804</td>
</tr>
<tr>
<td>Hex–EtOAc–H₂O 1–25–25</td>
<td>(K) 0.756</td>
<td>2.106 (2/1)</td>
<td>20.727</td>
<td>3.746</td>
</tr>
<tr>
<td>Hex–EtOAc–MeOH–H₂O 1–50–50</td>
<td>(K) 0.517</td>
<td>0.971 (2/1)</td>
<td>18.329</td>
<td>2.477</td>
</tr>
<tr>
<td>Hex–EtOAc–MeOH–H₂O 1–50–1–50</td>
<td>(K) 0.648</td>
<td>1.401 (2/1)</td>
<td>18.394</td>
<td>2.519</td>
</tr>
</tbody>
</table>

Abbreviations: MeOH, methanol; Hex, n-hexane; EtOAc, ethyl acetate.

and filtered through 0.45 \(\mu\)m membrane filter prior to injection into the HSCCC system.

2.4.3. HSCCC separation procedure

A multilayer coil of model TBE–300B HSCCC (Tauto Biotechnique Company, Shanghai, China) was used for the separation of cacao bean extract. The three preparative coils were connected in series \((\text{i.d. of the tubing} = 1.9 \text{ mm}, \text{total volume} = 300 \text{ mL})\) and equipped with a 20 mL sample loop. Both phases were pumped using a pump TBP–5002 (Tauto Biotechnique Company, Shanghai, China).

The column temperature was controlled at 25 \(^\circ\)C in the separation procedure. Both tail-head and head-tail elution were performed for the separation of cacao bean extract. The column was fitted with the lower stationary phase at a flow rate of 35 mL/min for a while and the upper mobile phase was pumped into the column with an optimal rotary speed of 950 rpm and flow rate of 3 mL/min in tail-head elution mode. Equilibration was achieved when the volume of the stationary phase in outlet end of the detector remained constant. Then 20 mL of the lower phase with 400 mg of cacao bean phenolic extract was injected through the sample loop to separate phenolic compounds. Retention of stationary phase was calculated. After the separation was carried out for 350 min, the mode was switched to the head-tail elution mode with the lower phase being pumped into the column. The effluents were monitored with a UV detector at \(\lambda\) 280 nm and the fractions were collected manually, followed by phenolic composition and structural identification in each of the fractions as described below.

2.5. Composition and structural identification of phenolic compounds in each fraction

Phenolic compounds in each of the fractions from HSCCC were identified by comparison with the standards through UPLC, MS analysis, \(^1\)H NMR analysis and further confirmed by phloroglucinolysis-UPLC analysis.

2.5.1. UPLC analysis

The phenolic composition of each isolated fractions from HSCCC was analyzed by an ACQUITY H-class UPLC, equipped with a Quaternary Solvent Manager (QSM), a Sample Manager with Flow through Needle (SM–FTN), and a Photodiode Array (PDA) Detector coupled to a data processing computer (EmpowerTM 2 chromatography data software). The column was an ACQUITY UPLC BEH C18 \((50 \times 2.1 \text{ mm}, 1.7 \text{ \mu m})\) and the temperature was set at 30 \(^\circ\)C. The flow rate of the mobile phase was fixed at 0.3 mL/min. The detection wavelength was set at 280 nm to monitor all phenolic compounds. Two elution solvents A \((\text{water: formic acid; 99.8: 0.2, } v/v)\) and B \((\text{acetonitrile: formic acid; 99.8: 0.2, } v/v)\) were used with the gradient elution program: 0 min \((A 98\%: B 2\%)\), 1 min \((A 96\%: B 4\%)\), 2 min \((A 88\%: B 12\%)\), 4 min \((A 88\%: B 12\%)\), 8 min \((A 68\%: B 32\%)\), 9 min \((A 0\%: B 100\%)\), 12 min \((A 98\%: B 2\%)\).

2.5.2. Phloroglucinolysis-UPLC analysis

Phloroglucinolysis-UPLC analysis was used for determination of structural composition and mean degree of polymerization \((mDP)\) of procyanidins in each of the isolated fractions from HSCCC, as well as for structural identification of individual procyanidins isolated from preparative HPLC. Phloroglucinolysis was performed based on the previous described method \([30]\) with some modifications. Briefly, 200 \(\mu\)L of fraction in methanol \((6 \text{ mg/mL})\) was added to 200 \(\mu\)L of phloroglucinol \((50 \text{ mg/mL})\) in methanol acidified by 0.2 M HCl in a stopped test tube. The tube was incubated for 20 min at 50 \(^\circ\)C for phloroglucinolysis reaction and then put in ice bath \((0 \text{ \degree C})\) to stop the reaction. The reaction mixture was filtered through 0.22 \(\mu\)m membrane filter prior to UPLC analysis. Calibration curves had been established in laboratory previous work \([30]\). Chromatographic conditions were the same as described in 2.5.1 Section.

2.5.3. MS analysis

Q-TOF MS analysis was used for identifying the composition of all the fractions except fraction 6 isolated from HSCCC. Analytical conditions were as follows: ion mode: negative; mass ranges: \(m/z 50–2000\); collision energy: 20–30 eV; capillary voltage: 2.5 kV; sample cone: 25 V; extraction cone: 4.0 V; source temperature: 130 \(^\circ\)C; desolation temperature: 400 \(^\circ\)C; cone gas flow rate: 50 L/h; desolation gas \((N₂)\) flow rate: 700 L/h.

The composition of fraction 6 was analyzed by LC/MS and the following conditions were used: ion mode: negative; mass ranges: \(m/z 150–2000\); sheath gas \((N₂)\): 35 arb and auxiliary gas \((N₂)\): 6 arb; capillary temperature: 300 \(^\circ\)C; capillary voltage: 25–30 V; tube lens offset voltage: 80 V; the ion spray voltage: 4.0 kV.

2.5.4. NMR analysis

One-dimensional \(^1\)H spectra analysis was performed on a Bruker ARX–600 MHz NMR Spectrometer. Methanol-\(d₄\) was used as solvent. The chemical shifts \((\delta)\) were calibrated against the residual solvent signals and are given in ppm. Coupling constants \((J)\) are given in Hz.
2.7.1. Scavenging

Monomers, dimers, trimers and tetrathers were isolated and purified from the fractions using preparative HPLC of waters Alliance e2695 Separation Module equipped with a 2998 Photodiode Array (PDA) Detector and coupled to a data processing computer (EmpowerTM 2 chromatography data software). Procyanidins were separated on a 250 x 10 mm, 5 μm, ODS-A column (YMC-Pack, Japan) and analysed at 280 nm. The column temperature was set at 30 °C. The flow rate of the mobile phase was fixed at 3 mL/min. Other chromatographic conditions were performed as follow: (--)-epicatechin and procyanidin B5 from Fraction 1, A70%: B30% (A: water; B: methanol); (+)-catechin and trimers I from Fraction 2, A79%: B21% (A: water; B: acetonitrile); procyanidin B2 from Fraction 3, A82%: B18% (A: water; B: methanol); procyanidin C1, tetramer I and II from Fraction 4, A85%: B15% (A: water; B: acetonitrile); theobromine and procyanidin C1 from Fraction 5, A70%: B30% (A: water; B: acetonitrile); procyanidin tetramer III and pentamer from Fraction 7 and 8, A87%: B13% (A: water; B: acetonitrile).

2.7. Antioxidant activity

The antioxidant capacity of each fraction from HSCCC, (--)-epicatechin, procyanidin B2 and procyanidin C1 was performed through three different methods measuring the free radical scavenging capacity (DPPH and ABTS assays) and Ferric reducing antioxidant power (FRAP assay).

2.7.1. DPPH assay

DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties. This method was conceptualised by Blois [31] in which the first time H atom accepting ability of a stable free radical DPPH• from cysteine molecule was demonstrated.

The scavenging effect of each fraction on DPPH was carried out as previously described method [32] with slight modifications. Briefly, DPPH solution was diluted with methanol to an absorbance of 0.66 (±0.02) at 517 nm. A 5 μL of tested sample solution or standard solution of trolox at different concentrations and 200 μL of DPPH solution were added directly to a 96 well microplate (Corning, America). The mixture reacted at room temperature in the dark for 60 min, then absorbance at 516 nm was recorded continuously against methanol as blank reference, using a microplate reader (Tecan Infinite M200 Pro, Switzerland) until the reaction reached the steady state. The samples were assayed in triplicate.

DPPH radical scavenging ability was calculated using Eq. (1):

\[
\text{Scavenging rate} (%) = \left( \frac{A_0 - A_i}{A_0} \right) \times 100
\]

(1)

where A0 and Ai are the absorbance of the control and the sample, respectively.

The antioxidant activity is expressed as EC50, which is defined as the amount of antioxidant needed to decrease the initial DPPH• concentration by 50%. And EC50 value was obtained by regression analysis from the dose–response curve which was performed by SPSS software (Version22.0, Chicago, IL, USA).

2.7.2. ABTS assay

The ABTS cation radical (ABTS•+) displays the exceptional property of a free radical to be relatively long-lived and stable for days [33], so that it can be applied as a useful tool for studying electron donation by radical scavengers.

The ABTS method was employed to measure radical scavenging activity of each fraction according to the method of Re et al. [33] with some modifications. ABTS was dissolved in phosphate buffered saline (PBS, pH 7.4) solution to a 7 mM concentration. ABTS radical cation (ABTS•+) was the products of equal amount of ABTS stock solution and 2.45 mM potassium persulfate mixing and reacting in the dark at room temperature for 12–16 h. The ABTS•+ solution was diluted with PBS to reach an absorbance of 0.66 ± 0.03 at 734 nm before use. A 10 μL of tested sample solution or standard solution at various concentrations and 200 μL of ABTS•+ solution were added directly to a 96 well microplate (Corning, America). The reaction was allowed to stand at room temperature for 10 min. Then the absorbance was measured at 734 nm against a blank reference of PBS using the same microplate reader as describe above. The samples were assayed in triplicate. The percentage of inhibiting ABTS•+ can also be calculated as Eq. (1) and the EC50 value was expressed as described above.

2.7.3. FRAP assay

Iron chelating ability of the samples were investigated using FRAP assay based on the reduction of Fe3+-TPTZ to a blue colored Fe2+-TPTZ. Ferric reducing antioxidant power (FRAP) assay developed by previous reported method [34] was performed with some modifications. Briefly, the FRAP working solutions was prepared at 37 °C and comprised of 50 mL of 300 mM acetate buffer (pH 3.6), 5 mL of 10 mM TPTZ solution in 40 mM HCl, and 5 mL of 20 mM FeCl3·6H2O solution.

A 180 μL amount of this solution was added in each well of 96 well microplate together with 5 μL of the samples or standards solution with a series of concentration and incubated at 37 °C for 10 min. Then the absorbance was measured at 593 nm, against a blank reference of water, in the same microplate reader. All measurements were done in triplicate. The standard curve was linear between 401 and 2003 μM FeSO4. FRAP value was expressed as μM FeSO4/μM sample under the same absorbance.

2.7.4. Statistical analysis

The antioxidant activity of each of the fractions from HSCCC was expressed as the EC50 value with the probit analysis and results were reported as means ± standard deviation (SD). The comparison of means was determined by one-way analysis of variance (ANOVA) with Duncan’s multiple range test. Correlations among data obtained were calculated using Pearson’s correlation coefficient (r). All statistical analyses were performed using SPSS.

3. Results and discussion

3.1. Optimization of HSCCC conditions

In order to achieve successful separation, solvent system should provide a suitable partition coefficient (K) to the analyte. Basically, K values between 0.5 and 2 are considered to be appropriate. If the K value is too low, the target peak will not be properly separated from other peaks, while the higher K value tends to reach a good resolution, but it will accompanied by broad peaks and a prolonged elution time [27,35]. Meanwhile, the separation factor (α) between the target compounds which is another crucial factor for the successful separation ought to be greater than 1.5 [27,36].

In this work, in order to obtain a suitable solvent system and elution mode for the separation of cacao bean extract, six solvent systems were selected, and K values and the separation factor (α) of (--)–epicatechin, OPCS, PPCs and cacao bean extract are presented in Table 1.

As is seen in Table 1, all the α values were greater than 1.5, indicating that the separation resolution of these solvent systems all are suitable. The K values of (--)–epicatechin in n-Butanol–EtOAc–H2O (1–20–20, v/v/v), MeOH–EtOAc–H2O (1–25–25, v/v/v) and EtOAc–H2O (1–1, v/v) systems was less than 0.5, and the K values of OPC in Hex–EtOAc–H2O (1–20–20, v/v/v) was higher
than 2, which were not feasible for separation. Theoretically, the K values in the Hex–EtOAc–H2O (1–50–50, v/v) system and Hex–EtOAc–MeOH–H2O (1–50–1–50, v/v) indicated that the two systems were both suitable for separation, however, the retention rate and resolution of the latter was lower than the former. As a result, the solvent system of Hex–EtOAc–H2O (1–50–50, v/v) was selected for this study. (−)-Epicatechin and OPCs could be eluted from HSCCC column in tail-head elution mode with this solvent system, however, PPCs could not be eluted from column in tail-head elution mode with the upper mobile phase due to the higher K value, the head-tail elution mode with the lower mobile phase was applied for the separation of PPCs.

The flow rate of the mobile phase and sample load were also optimised. The flow rate at 3.0 mL/min and 3.5 mL/min were investigated. And sample load was investigated from 300 to 600 mg of the cacao bean phenolic extract in 20 mL of the lower phase. The high flow rate tended to give poor separation resolution and large amounts of sample would lead to a great loss of stationary phase from the column. Finally, the flow rate was set to 3.0 mL/min and sample load was 400 mg. With the optimised condition, higher yield could be achieved and the peaks of the chromatography reached a well separation, and the retention of the stationary phase was up to 55% calculated by the volume of stationary phase in the column divided by the total volume of the column.

Thus, on the basis of above HSCCC optimized conditions, cacao bean phenolic extract could be separated into 8 fractions (Fig. 1). By each run. The yields of fractions collected by HSCCC separation were also very acceptable, 82.8 mg of Fraction 1, 9.2 mg of Fraction 2, 23.5 mg of Fraction 3, 18.6 mg of Fraction 4, 25 mg of Fraction 5, 104 mg of Fraction 6, 59.2 mg of Fraction 7 and 57.4 mg of Fraction 8 were obtained from 400 mg of sample loading. Furthermore, the phenolic compositions and antioxidant activity of these fractions are very different, which are described below.

3.2. Structural characterization of the fractions from HSCCC by phloroglucinolysis–UPLC

The structural characterization of procyanidins in each of the fractions from HSCCC was determined by phloroglucinolysis–UPLC. Phloroglucinolysis–UPLC analysis was a useful tool for structural characterization of procyanidins, which permitted to quantify the terminal units (released as monomeric flavan-3-ols) and extension units (released as phloroglucinol derivatives) as well as mean degree of polymerization (mDP) [22]. The compositional data, together with mean polymerization degree (mDP) of proanthocyanidins in Fraction 1 to 8 isolated from HSCCC of cacao bean phenolic extract are presented in Table 2.

It can be noted that although mDP was not strictly in increasing order, Fraction 1 presented the lowest mDP, being 1.58; the values of mDP for Fraction 2, 3 and 5 were 2–3, while the Fraction 4 and 6–8 presented mDP around 4 to 5.

Neither (−)-epicatechin-3-O-gallate nor (−)-epicatechin-3-O-galloyl-phloroglucinol derivative existed in any fractions. Fraction 1–5 were comprised of (+)-catechin and (−)-epicatechin both as extension and terminal units, however, Fraction 6–8 did not detected (+)-catechin as terminal unit under hydrolyzation of phloroglucinol. The extension and terminal units exerted multiple distribution characteristics in different fractions. (−)-Epicatechin was both the terminal unit and extension unit with the quantitatively predominant in each fraction and the percentage in moles ranged from 21.49% to 56.44%, 34.52% to 77.17%, respectively.

The mDP of Fraction 1 was 1.58, corresponding to the composition of (−)-epicatechin and dimer. The mDP of Fraction 2 was 2.87, conformed to the existing of (+)-catechin and trimer detected by UPLC. For Fraction 3 and 5, the mDP were 2.58 and 3.83, which meant they were mainly contained dimer and trimer, respectively. The mDP of Fraction 4 and 6–8 were all more than 4, which indicated that these factions were comprised of higher oligomeric procyanidins than any other factions and it was consistent to the results that tetrarmers were detected in each of these fractions as well as pentamers in Fraction 7 using UPLC and MS analysis.

3.3. UPLC analysis and preparative isolation of individual procyanidins

Individual phenolic compounds in each fraction separated from HSCCC were verified by UPLC and the major individual procyanidins were further isolated using HPLC in a preparative scale.

3.3.1. Fraction 1

It can be seen from the chromatogram of UPLC analysis (Fig. 2A) that the polyphenols in Fraction 1 were composed essentially of (−)-epicatechin, together with lower amounts of (+)-catechin and dimeric procyanidin B5. Based on these results, the large separation of (−)-epicatechin, (+)-catechin and procyanidin B5 were performed by preparative HPLC (Fig. 2B). As a result, the three phenolic compounds could be successfully isolated one another. Each run of HPLC with injection of 82.8 mg of Fraction 1 could yield 61.28 mg of (−)-epicatechin, 4.14 mg of procyanidin B5 with HPLC purity over 95%. The purity of these compounds were verified by HPLC–DAD and LC–MS. (−)-Epicatechin from other origin are commercially available, while dimeric procyanidin B5 are not. The present work developed a new and rapid procedure for large isolation of these compounds by HSCCC combined with preparative HPLC.

3.3.2. Fraction 2

It can be seen from the chromatogram of UPLC analysis (Fig. 2C) that the polyphenols in Fraction 2 were composed essentially of (+)-catechin, together with lower amounts of trimeric procyanidin T3. Based on these results, the large separation of (+)-catechin and procyanidin T3 were performed by preparative HPLC (Fig. 2D). As a result, the two procyanidins compounds could be successfully isolated from one another. Each run of HPLC with injection of 9.2 mg of Fraction 2 could yield 4.6 mg of (+)-catechin with HPLC purity over 98% and 1.65 mg of procyanidin T3 with HPLC purity over 95%. HPLC–DAD and LC–MS were used for verifying the purity of each compound.
Fig. 2. Chromatograms of UPLC analysis (A) and preparative HPLC (B) of Fraction 1; Chromatograms of UPLC analysis (C) and preparative HPLC (D) of Fraction 2; chromatograms of UPLC analysis (E) and preparative HPLC (F) of Fraction 3; Chromatograms of UPLC analysis (G) and preparative HPLC (H) of Fraction 4.
Table 2
Structural characterization and mean polymerization degree (mDP) of HSCCC fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Terminal units (percent in moles)</th>
<th>Extension units (percent in moles)</th>
<th>mDP ± δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>EC</td>
<td>CP</td>
</tr>
<tr>
<td>1</td>
<td>6.67 ± 0.06</td>
<td>56.44 ± 0.10</td>
<td>2.36 ± 0.00</td>
</tr>
<tr>
<td>2</td>
<td>1.88 ± 0.02</td>
<td>32.97 ± 0.21</td>
<td>2.88 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>2.27 ± 0.13</td>
<td>36.43 ± 0.11</td>
<td>1.63 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>2.43 ± 0.02</td>
<td>21.85 ± 0.15</td>
<td>1.28 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>2.63 ± 0.03</td>
<td>23.51 ± 0.10</td>
<td>2.11 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>21.76 ± 0.12</td>
<td>18.51 ± 0.27</td>
</tr>
<tr>
<td>7</td>
<td>N.D.</td>
<td>21.49 ± 0.04</td>
<td>1.09 ± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>23.45 ± 0.00</td>
<td>1.59 ± 0.02</td>
</tr>
</tbody>
</table>

Abbreviations: C, (+)-catechin; EC, (-)-epicatechin; CP, (+)-catechin-phloroglucinol derivative; ECP, (-)-epicatechin-phloroglucinol derivative; τ, mean concentration; δ, standard deviation. N.D., not determined.

3.3.3. Fraction 3
It can be seen from the chromatogram of UPLC analysis (Fig. 2E) that dimer B2 in Fraction 3 could be obtained by one step HSCCC separation of cacao bean extract, and the purity was over 86%. After further purification by preparative HPLC (Fig. 2F), each injection of 23.5 mg of Fraction 3 could yield 15.27 mg of dimer B2 with HPLC purity over 98%. UPLC–DAD and LC–MS were employed to confirm the purity of procyandin B2. From the proposed one step HSCCC method, large scale preparation of dimer B2 could be rapidly achieved.

3.3.4. Fraction 4
It can be seen from the chromatogram of UPLC analysis (Fig. 2G) that the polyphenols in Fraction 4 were composed essentially of trimer C1, together with lower amounts of two tetrameric procyanidins. Furthermore, the large separation of trimer C1 and tetramer I were performed by preparative HPLC (Fig. 2H). As a result, the three phenolic compounds could be successfully isolated one another. Each run of HPLC with injection of 8.6 mg of Fraction 4 could yield 8.74 mg of trimer C1, 1.86 mg of tetramer I with HPLC purity over 95%. HPLC–DAD and LC–MS were used for checking the purity of each compound. The result indicated that the procedure of HSCCC separation of cacao bean extract could be a supplementary means for large preparation of trimer C1.

3.3.5. Fraction 5
It can be seen from the chromatogram of UPLC analysis (Fig. 3A) that the polyphenols in Fraction 5 were composed essentially of theobromine and trimeric procyanidin C1. Both of these polyphenols were further isolated by preparative HPLC under the optimized elution conditions (Fig. 3B). Each run of HPLC with injection of 25 mg of Fraction 5 could yield 4.3 mg of trimer C1 with HPLC purity over 95%. The purity of this compound was verified by HPLC–DAD and LC–MS.

3.3.6. Fraction 6
It can be seen from the chromatogram of UPLC analysis (Fig. 3C) that the polyphenols in Fraction 6 were composed of little tetramer, pentamer and hexamer. However, these three procyanidins were hardly largely isolated by preparative HPLC. This might be because that there was trace amount of procyandin in the fraction and the lower solubility was interfered by other phenolic compounds.

3.3.7. Fraction 7
It can be seen from the chromatogram of UPLC analysis (Fig. 3D) that the polyphenols in Fraction 7 were composed essentially of tetramer, pentamer and an unknown compound which m/z was 294 [M–H]− with fragment ions m/z 276, 132 and 115. Furthermore, the large separation of procyandinis tetramer III and pentamer were performed by preparative HPLC (Fig. 3E). As a result, the two phenolic compounds could be successfully isolated from one another. Each run of HPLC with injection of 59.2 mg of Fraction 7 could yield 21.9 mg of tetramer III, 12.43 mg of pentamer with HPLC purity over 95%. HPLC–DAD and LC–MS were used for checking the purity of each compound. Because tetramer and pentamer is not commercially available, this work provided a feasible method of the preparation of high purity tetramer using HSCCC technology combined with preparative HPLC.

3.3.8. Fraction 8
It can be seen from the chromatogram of UPLC analysis (Fig. 3F) that the main polyphenols in Fraction 8 were the same type of tetramer and an unknown compound as described in Fraction 7. Furthermore, the large separation of tetramer III was performed by preparative HPLC (Fig. 3G). Each run of HPLC with injection of 57.4 mg of Fraction 8 could yield 11.48 mg of tetramer III with HPLC purity over 95%. HPLC–DAD and LC–MS were used for verifying the purity of this compound.

3.4. Structural identification
The procyandinis obtained by HSCCC or preparative HPLC as described above were identified by comparison with the standards and literature through MS analysis [30], phloroglucinolysis-UPLC analysis (as shown in Table 3), as well as 1H NMR when necessary.

3.4.1. (+)-Catechin
UPLC retention time (4.14 min), identical to that of the standard; MS analysis: m/z 289 ([M–H]−), 245, 139, 137 (MS2 ions).

3.4.2. (-)-Epicatechin
UPLC retention time (5.28 min), identical to that of the standard; MS analysis: m/z 289 ([M–H]−), 245, 139, 137 (MS2 ions).

3.4.3. Procyanidin B5
MS analysis: m/z, 577 ([M–H]−), 559, 451, 425, 407, 289 (MS2 ions); Phloroglucinolysis-UPLC: terminal unit, (-)-epicatechin; extension unit, (-)-epicatechin. UPLC retention time (7.22 min) was later than any other procyanidins, and it was in accordance with the results of Esatbeyoglu et al. [14].

3.4.4. Procyanidin T3
MS analysis: m/z, 865 ([M–H]−), 847, 739, 713, 695, 577, 425 (MS2 ions); Phloroglucinolysis-UPLC: terminal unit, (-)-epicatechin; extension unit, two (-)-epicatechin. Considering that all naturally-occurring oligomers in T. cacao were composed of (-)-epicatechin units which were linked via a C4 –C8 bond in
Fig. 3. Chromatograms of UPLC analysis (A) and preparative HPLC (B) of Fraction 5; Chromatograms of UPLC analysis (C) of Fraction 6; Chromatograms of UPLC analysis (D) and preparative HPLC (E) of Fraction 7; Chromatograms of UPLC analysis (F) and preparative HPLC (G) of Fraction 8.
Table 3  
UPLC retention time ($t_R$) of main individual procyanidins, their major ions observed in the MS/MS spectra and their corresponding terminal and extension units.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_R$ (min)</th>
<th>$[M-\text{H}]^-$</th>
<th>MS² ions (m/z)</th>
<th>Terminal unit</th>
<th>Extension unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.14</td>
<td>289</td>
<td>245, 179, 137</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>EC</td>
<td>5.28</td>
<td>289</td>
<td>245, 179, 137</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>B5</td>
<td>7.22</td>
<td>577</td>
<td>559, 451, 425, 407, 289</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>T3</td>
<td>7.08</td>
<td>865</td>
<td>847, 739, 713, 695, 577, 425</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>C1</td>
<td>5.72</td>
<td>865</td>
<td>847, 739, 713, 695, 577, 425, 407</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>Tetramer I</td>
<td>6.26</td>
<td>1153</td>
<td>1027, 865, 739, 701, 577, 289</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>Tetramer II</td>
<td>6.39</td>
<td>1153</td>
<td>1027, 865, 739, 701, 577, 289</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>Tetramer III</td>
<td>6.00</td>
<td>1141</td>
<td>1027, 865, 739, 701, 577, 289</td>
<td>EC</td>
<td>EC</td>
</tr>
<tr>
<td>Pentamer</td>
<td>6.18</td>
<td>1441</td>
<td>1153, 865, 720, 305</td>
<td>EC</td>
<td>EC</td>
</tr>
</tbody>
</table>

Abbreviations: C, (+)-catechin; EC, (−)-epicatechin; B5, procyanidin dimer B5; T3, procyanidin trimmer T3; C1, procyanidin trimer C1; $t_R$, retention time.

Table 4  
Antioxidant activity and linear range of isolated fractions and individual procyanidins by DPPH, ABTS and FRAP assays.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH (µmol/L)</th>
<th>ABTS (µmol/L)</th>
<th>FRAP (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀</td>
<td>Linear range</td>
<td>EC₅₀</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>189.5 ± 2.1c</td>
<td>33–330</td>
<td>50.0 ± 0.0d</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>143.6 ± 5.0d</td>
<td>15–152</td>
<td>39.3 ± 1.2e</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>143.0 ± 12.7d</td>
<td>20–200</td>
<td>36.7 ± 0.6f</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>97.0 ± 0.0ef</td>
<td>13–126</td>
<td>26.0 ± 0.0i</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>92.0 ± 1.7e</td>
<td>11–108</td>
<td>22.0 ± 0.0j</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>196.0 ± 15.6c</td>
<td>25–285</td>
<td>53.0 ± 1.4c</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>297.0 ± 17.0b</td>
<td>52–519</td>
<td>75.3 ± 1.5h</td>
</tr>
<tr>
<td>B2</td>
<td>139.7 ± 3.5d</td>
<td>21–208</td>
<td>32.5 ± 2.1g</td>
</tr>
<tr>
<td>C1</td>
<td>114.7 ± 4.1e</td>
<td>18–182</td>
<td>28.7 ± 0.6h</td>
</tr>
<tr>
<td>Trolox</td>
<td>802.3 ± 13.7a</td>
<td>204–1020</td>
<td>509.0 ± 3.3a</td>
</tr>
</tbody>
</table>

Abbreviations: EC₅₀ values or FRAP values were presented as mean ± standard deviation. Different letters in a column mean significant differences, p < 0.05.

B-type [14], the compound was inferred (−)-epicatechin (4β, 6)− (−)-epicatechin (4β, 8), (−)-epicatechin, namely, procyanidin T3.

3.4.5. Procyanidin B2

UPLC retention time (4.69 min), identical to that of the standard; MS analysis: $m/z$, 577 ([M−H]⁻), 559, 451, 425, 407, 289 (MS² ions; Phloroglucinolysis-UPLC: terminal unit, (−)-epicatechin; extension unit, (−)-epicatechin.

3.4.6. Procyanidin C1

MS analysis: $m/z$, 865 ([M−H]⁻), 847, 739, 713, 695, 577, 425, 407 (MS² ions); Phloroglucinolysis-UPLC: terminal unit, (−)-epicatechin; extension unit, two (−)-epicatechin; ¹H NMR (600 MHz, Methanol-d₄): 2.84 (d, $J = 16.5$ Hz, H-4α), 2.97 (d, $J = 16.8$ Hz, H-4β), 4.05 (d, $J = 13.2$ Hz, H-3α), 3.31, 4.34 (s, br s, H-3β), 4.74 (s, 2H, H-4α), 5.09 and 5.25 (2 br s, H-2α, H-2β, H-2γ), 5.97 and 6.06 (m, H-6γ, 8 γ, 6 M), 6.69–6.85 (m, H-5′, 5′γ, 6′, 6′γ, 6′, 6′γ, 5′γ, 5′α, 5′α). On the basis of the data described above and the verified reference [37,38], the structure of the compound was considered as procyanidin C1.

The scavenging capacities of the HSCCC fractions and three individual procyanidins ((−)-epicatechin, procyanidin B2 and procyanidin C1) on DPPH* expressed as EC₅₀ values with trolox as control. A dose-dependent relationship was found in DPPH* scavenging activity within the range of concentrations listed in Table 4. The EC₅₀ values indicated that both the fractions and three individual procyanidins had rather strong activity for radical scavenging than trolox. The scavenging capacities on DPPH* of the individual procyanidins were found to be in decreasing order of procyanidin C1 > procyanidin B2 > (−)-epicatechin. All of the HSCCC fractions tested exhibited more powerful DPPH* scavenging activities than (−)-epicatechin. Basically, the antioxidant capacity enhanced with the increase of their mean polymerization degree except Fraction 8 which might be influenced by the little amount of procyanidins and other polyphenols. The results suggested that the scavenging effect on DPPH free radical was affected by not only the mean polymerization degree, but also the polarity of polyphenols which determine their existing form (free or bonded) in the medium of reaction. Other bioactive compounds in the fractions such as methylxanthine (theobromine) might interfered with the reaction and absorbance as well.

ABTS and FRAP assays indicated the similar tendency with the DPPH assay. The HSCCC fractions were found to exhibit ABTS** scavenging activities higher than trolox and monomer (−)-epicatechin. Their activities in dose dependent manner and the EC₅₀ values were represented at Table 4. The FRAP value of the fraction was positives related with ferric reducing antioxidant power and was negatively correlated with DPPH and ABTS assays.

The correlations of antioxidant activities based on ABTS, DPPH and FRAP assays were estimated using Pearson’s correlation coefficient (2-tailed). The high correlations were found among assays. The highest correlations was found between ABTS and DPPH assays (r = 0.984, P < 0.001) and the lowest correlations between FRAP and
Conflict of interest

The authors have declared no conflict of interest.

References


ABTS (r = −0.661, P = 0.038). DPPH assay was positively highly correlated with ABTS assay, while FRAP assay was negatively highly correlated with DPPH and ABTS assays, especially between FRAP and DPPH (r = −0.774, P = 0.009).

4. Conclusion

Under the optimized conditions, HSCCC could successfully separate, in a preparative scale, cocoa bean polyphenols into several distinct fractions containing procyanidins ranging from monomer up to pentamer. Further large separation of the major individual procyanidins in these fractions could be achieved by preparative HPLC. Procyanidin dimer B2 and B5, trimer C1 and T3, tetramers and pentamer were isolated from cacao beans. Finally, both the isolated fractions and individual procyanidins of cacao bean extract were verified to possess potential antioxidant activities.