Efficient Extraction of Bioactive Flavonoids from *Ginkgo biloba* Leaves Using Deep Eutectic Solvent/Water Mixture as Green Media

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Deep eutectic solvent (DES)/water mixture as alternative extraction solvent was proposed for the efficient extraction of Ginkgo flavonoids from *Ginkgo biloba* leaves. Fifty DESs were prepared and investigated for the extraction of Ginkgo flavonoids. Compared with the present most efficient extraction solvent (70 % ethanol in water), three DESs, choline chloride/1,3-butanediol (ChCl/B), choline chloride/levulinic acid (ChCl/LA1), and 1,2-propanediol/levulinic acid (P/LA1), gave obviously higher extraction yields. The extraction process was further optimized systematically. The optimized extraction conditions were as follows: ChCl/LA1 containing 40 % (w/w) water was used as the solvent to extract Ginkgo flavonoids at a solvent to solid ratio of 10:1 (v/w) with stirring at 50 °C and 150 rpm for 15 min. Under the optimal conditions, 99.87 % of Ginkgo flavonoids could be extracted from the *Ginkgo biloba* leaves powder at a time. Furthermore, the recovery of Ginkgo flavonoids in the DES extraction solution was efficiently achieved using macroporous resin AB-8, which gave a recovery yield of 93.7 %. The DES-based extraction combined with macroporous resin recovery developed in this work can be an efficient alternative method for the extraction and separation of Ginkgo flavonoids from *Ginkgo biloba* leaves.

Keywords: deep eutectic solvents, *Ginkgo biloba*, Ginkgo flavonoids, extraction, recovery

Introduction

*Ginkgo biloba* is one of the oldest known trees on earth with fossil records dating back more than 200 million years. It survived from the era when dinosaurs became extinct. Its amazing vitality has attracted increasing exploration into its potential application in health foods and supplements. *Ginkgo biloba* has been used in traditional Chinese medicine for thousands of years, and it is helpful in inhibiting the onset of dementia, slowing down cognitive decline, and reducing the incidence of cardiovascular disease because of its ability to prevent free radical damage, support microcirculation, and improve brain function.

*Ginkgo biloba* leaves contain various species of active ingredients, for example ginkgolides, bilobalide, flavonoids, proanthocyanidins, alkylphenols, simple phenolic acids, and so on. Ginkgo flavonoids are the most important class of compounds in *Ginkgo biloba* leaves. About 38 different flavonoids have been isolated from *Ginkgo biloba* leaves, and most of them are multiform glycosides of quercetin, kaempferol and isorhamnetin. Ginkgo flavonoids are believed to act as protectants against capillary fragility, anti-inflammatory agents, and antioxidants, in reducing edema caused by tissue injury, and as free radical scavengers.

Because of the potentials of the Ginkgo flavonoids as novel drugs and healthcare products, there is a high annual demand for the large-scale production of flavonoids. Preparative HPLC method has been extensively employed in the isolation of flavonoids, and many references have reported about this technology. However, it is not suitable for industrial scale preparation. For many years, liquid extraction using different organic solvents has been widely adopted by most manufacturers. Ethanol, methanol and acetone are the most commonly used organic solvents. As is well-known, conventional organic solvents are responsible for environmental pollution because of their inflammability and volatility.
Ginkgo flavonoids can also be obtained using supercritical CO₂ extraction technology (SFE)⁹–¹¹. In contrast with conventional extraction processes using organic solvents, SFE is an ideal alternative to deal with heat-sensitive or easily oxidizable material. However, dry supercritical CO₂ cannot extract flavonoids effectively, and thus addition of a cosolvent such as ethanol is needed⁸–¹¹. Furthermore, the SFE is still in the laboratory-scale experimental stage.

In recent years, deep eutectic solvent (DES) has attracted increasing attention as an excellent alternative to the conventional organic solvents¹²–¹³. DESs are made up by mixing two or three components, which are capable of self-association through hydrogen bond interactions, to form eutectic mixtures having melting points lower than that of each individual component¹⁴. They are mostly formed by mixing quaternary ammonium salts with a range of organic acids, saccharides and amino acids¹⁵–¹⁷. Compared with conventional organic solvents, DESs possess many preferable characteristics, including safety, non-toxicity, biodegradability, sustainability, low cost, and easy preparation. Moreover, they show good physicochemical properties: adjustable viscosity, negligible volatility, wide polarity range, and high dissolving capacity for a variety of compounds¹⁸. Actually, DESs have been applied in catalysis, organic synthesis, dissolution, electrochemistry and material chemistry, aiming at increasing efficiency and reducing pollution¹⁵–¹⁷,¹⁹. There are also reports on the applications of DESs for the extraction of proteins²⁰, phenolic compounds²¹,²², anthocyanin²³, astaxanthin²⁴, ginseng saponins²⁵, catechins²⁶, flavonoids²⁶–²⁹, and many more applications are continuously being explored.

In this work, DESs were used as the alternative solvents to extract Ginkgo flavonoids from Ginkgo biloba leaves. The parameters relevant to the extraction efficiency due to the DESs conditions (hydrogen bond acceptors (HBAs), HBDs, HBA/HBD ratio, and water content) and extraction conditions (extraction method, temperature, DES to solid ratio and time) were examined systematically.

Materials and methods

Materials

The Ginkgo biloba leaves used in this study were bought from Chinese Herb Transaction Center (Bozhou, China). The leaves were dried at 65 °C to constant weight, and then pulverized by a disintegrator. The pulverized material was sieved between 30- and 40-mesh, and then stored in a desiccator prior to use.

All compounds of analytical reagent grade used for DESs preparation were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China) and used without further purification. HPLC-grade methanol was obtained from Tedia Company, Inc., (Shanghai, China). HPLC-grade phosphoric acid was bought from J & K Chemical Ltd. (Beijing, China). Deionized water was obtained by a Milli-Q water purification system (Millipore, Billerica, MA). The standards of quercetin, kaempferol and isorhamnetin were purchased from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China), and their purities were more than 98 %. All other reagents and chemicals used in the experiment were of analytical reagent grade. All samples were filtered using a filter (0.22 μm) before being injected into the HPLC system.

DESs preparation

DESs were prepared following the method described by Abbot et al.²⁰ In a typical procedure, component 1 and component 2 were mixed and heated between 80 °C and 110 °C at selected molar ratios with constant stirring until homogeneous and transparent liquids were formed. If the mixture formed turbid liquid after heating for 3 h at 110 °C, it indicated that it could not form a DES at selected ratio. The DESs prepared in this work are listed in Table 1.

Extraction of flavonoids from Ginkgo biloba leaves and hydrolysis of the extract

For the initial DES screening, an accurately weighed 50-mg sample of Ginkgo biloba leaves powder was mixed with 0.75 mL of extraction solvent in a 2.0-mL microfuge tube. After brief vortexing, the mixture was extracted in the ultrasonic facilities (XO-5200DTNSN, Nanjing Sinotech Co., Ltd., China) with an ultrasound-assisted program at 100 W and 60 °C for 60 min, and then centrifuged at 10,000 rpm for 30 min. Parent flavonoid glycosides in the supernatant were converted to their respective aglycons by acid hydrolysis, during which 300 μL of the supernatant mixed with 500 μL of 1.5 M hydrochloric acid methanol solution was heated at 90 °C for 40 min. The hydrochloric acid concentration and time for acid hydrolysis were obtained by optimization (data not shown). The extraction yield of total flavonoids was assessed based
on the flavonoid aglycon levels determined by HPLC analysis.

**DESs extraction of Ginkgo flavonoids employing different methods**

After initial DES screening, three DESs (ChCl/B, ChCl/LA1 and P/LA1) were chosen to investigate the extraction efficiency of different extraction methods. Heating, stirring, heating + stirring, and ultrasonic methods were compared for the extraction. A 50-mg sample of *Ginkgo biloba* leaves powder was mixed with 0.75 mL of the three DESs containing 30 % (w/w) of deionized water. The mixture was extracted for 10 min by heating at 60 °C and 0 rpm, stirring at 25 °C and 150 rpm, heating + stirring at 60 °C and 150 rpm, or ultrasonic treating at 50 W and 25 °C, respectively.

**Optimization of extraction conditions**

The stirring method was selected for the further optimization of extraction parameters. The parameters included water content in DES (0, 10, 20, 30, 40, 60, 80 and 100 %, w/w), extraction temperature (25, 30, 35, 40, 45, 50, 55 and 60 °C), ratio between DES volume and *Ginkgo biloba* leaves powder weight (7.5:1, 10:1, 12.5:1, 15:1, 20:1, 30:1, and 50:1, mL g⁻¹), and extraction time (5, 10, 15, 20, 25, 30 and 40 min).

**Recovery of Ginkgo flavonoids from the DES extraction solution**

The recovery of the target Ginkgo flavonoids from DES extraction solution was carried out by adsorption using different macroporous resins. An amount of 5.0 mL of DES extraction solution was put into a 10-mL flask, and 2.0 g macroporous resin was then added. The adsorption was operated at 25 °C and 150 rpm for 6 h. The macroporous resin was filtered out, and then desorbed with 5.0 mL of 95 % (v/v) ethanol at 25 °C and 150 rpm for 4 h. The Ginkgo flavonoids content in the DES extraction solution, the solution after adsorption, and the solution after desorption were determined separately. Accordingly, the adsorption yield of macroporous resin and the desorption yield of 95 % (v/v) ethanol were calculated. The recovery process was performed in triplicate, and the results expressed as the mean.

**HPLC analysis of flavonoid aglycons**

HPLC analysis was carried out on a Waters HPLC 2695 system (Waters, USA) equipped with a Sino Chrom ODS-BPC18 column (4.6×200 mm, 5 µm; Elite, China), and a 2489 UV detector at 360 nm. Mobile phase was a mixture of methanol and 0.05 % phosphoric acid solution (57:43, v/v). The injection volume was maintained at 30 °C, and the flow rate was set at 1.0 mL min⁻¹ with the injection volume of 10 µL.

The extraction yield of total flavonoids was assessed based on the flavonol aglycon levels (as glycosides), which were calculated from the measured quantities of flavonol aglycons (quercetin, kaempferol and isorhamnetin) following acid hydrolysis. After acid hydrolysis, the solution was diluted appropriately with the mobile phase, and filtered through a 0.22 µm membrane filter before being injected into the HPLC system. The factors for conversion from aglycon mass to glycoside mass are 2.51 for quercetin, 2.64 for kaempferol, and 2.39 for isorhamnetin. In that case, the mass of flavonoids extracted from *Ginkgo biloba* leaves (m_flavonoids) was calculated by:

\[
m_{\text{flavonoids}} = 2.51 \cdot m_{\text{quercetin}} + 2.64 \cdot m_{\text{kaempferol}} + 2.39 \cdot m_{\text{isorhamnetin}}.
\]

The extraction yield of total flavonoids obtained was expressed as:

\[
y_{\text{extraction}} = \frac{m_{\text{flavonoids}}}{m_{\text{leaf}}} \times 100\%.
\]

**Results and discussion**

**Preparation of various types of DESs**

DES can be prepared from combinations of two components at various molar ratios using heating, evaporating, or freeze-drying method. In this work, the heating method was used because its procedure is simple. The main criteria for DES component selection in the present work were low cost, safety, and good biodegradability. Based on previous reports and our own experience, a number of components were used with the aim of forming DESs at various ratios. Choline chloride, as the most used HBA, was firstly chosen to form DESs with different alcohols, sugars, organic acids, and urea. Fifteen combinations were initially tested. As shown in Table 1 (Entries 1–15), 10 combinations were found to be stable as clear liquids, 4 combinations were found to be viscous liquids, and 1 combination formed a transparent gel. Further, betaine and proline were used to replace the choline chlo-
| Entry | Abbreviation | Component 1 | Component 2 | Molar ratio | Appearance               |
|-------|--------------|-------------|-------------|-------------|--------------------------|
| 1     | ChCl/G       | Choline chloride | Glycerol  | 1:2         | Clear liquid             |
| 2     | ChCl/EG      | Choline chloride | Ethylene glycol | 1:2      | Clear liquid             |
| 3     | ChCl/P       | Choline chloride | 1,2-Propanediol | 1:2   | Clear liquid             |
| 4     | ChCl/B       | Choline chloride | 1,3-Butanediol | 1:3    | Clear liquid             |
| 5     | ChCl/DS      | Choline chloride | D-Sorbitol | 1:1     | Viscous and clear liquid |
| 6     | ChCl/DG      | Choline chloride | D-Glucose | 1:1     | Transparent gel          |
| 7     | ChCl/GA1     | Choline chloride | Glutaric acid | 1:1   | Clear liquid             |
| 8     | ChCl/GA2     | Choline chloride | Glycolic acid | 1:1  | Clear liquid             |
| 9     | ChCl/MA1     | Choline chloride | Malonic acid | 1:1   | Clear liquid             |
| 10    | ChCl/MA2     | Choline chloride | Malic acid | 1:1   | Viscous and clear liquid |
| 11    | ChCl/LA1     | Choline chloride | Levulinic acid | 1:2 | Clear liquid             |
| 12    | ChCl/LA2     | Choline chloride | Lactic acid | 1:1   | Clear liquid             |
| 13    | ChCl/CA      | Choline chloride | Citric acid | 1:1   | Viscous and clear liquid |
| 14    | ChCl/TA      | Choline chloride | L-(+)-Tartaric acid | 2:1 | Viscous and clear liquid |
| 15    | ChCl/U       | Choline chloride | Urea | 1:2  | Clear liquid             |
| 16    | BE/G         | Betaine | Glycerol | 1:2 | Clear liquid             |
| 17    | BE/EG        | Betaine | Ethylene glycol | 1:3 | Clear liquid             |
| 18    | BE/P         | Betaine | 1,2-Propanediol | 1:3 | Clear liquid             |
| 19    | BE/B         | Betaine | 1,3-Butanediol | 1:3 | Clear liquid             |
| 20    | BE/X         | Betaine | Xylitol | 1:2 | Viscous and clear liquid |
| 21    | BE/DS        | Betaine | D-Sorbitol | 1:2 | Viscous and clear liquid |
| 22    | BE/GA2       | Betaine | Glycolic acid | 1:1 | Clear liquid             |
| 23    | BE/MA1       | Betaine | Malonic acid | 1:2 | Viscous and clear liquid |
| 24    | BE/OA        | Betaine | Oxalic acid | 1:1 | Clear liquid             |
| 25    | BE/MA2       | Betaine | Malic acid | 1:1 | Viscous and clear liquid |
| 26    | BE/LA1       | Betaine | Levulinic acid | 1:2 | Clear liquid             |
| 27    | BE/LA2       | Betaine | Lactic acid | 1:1 | Clear liquid             |
| 28    | BE/CA        | Betaine | Citric acid | 1:1 | Viscous and clear liquid |
| 29    | BE/TA        | Betaine | L-(+)-Tartaric acid | 2:1 | Unable to form clear liquid |
| 30    | PR/G         | Proline | Glycerol | 1:1 | Unable to form clear liquid |
| 31    | PR/P         | Proline | 1,2-Propanediol | 1:1 | Unable to form clear liquid |
| 32    | PR/B         | Proline | 1,3-Butanediol | 1:1 | Unable to form clear liquid |
| 33    | PR/X         | Proline | Xylitol | 1:1 | Unable to form clear liquid |
| 34    | PR/DS        | Proline | D-Sorbitol | 1:1 | Unable to form clear liquid |
| 35    | PR/MA2       | Proline | Malic acid | 1:1 | Viscous and clear liquid |
| 36    | PR/GA1       | Proline | Glutaric acid | 1:1 | Unable to form clear liquid |
| 37    | PR/GA2       | Proline | Glycolic acid | 1:1 | Clear liquid             |
| 38    | PR/LA1       | Proline | Levulinic acid | 1:1 | Unable to form clear liquid |
| 39    | PR/LA2       | Proline | Lactic acid | 1:1 | Clear liquid             |
| 40    | PR/CA        | Proline | Citric acid | 1:1 | Viscous and clear liquid |
| 41    | B/LA2        | 1,3-Butanediol | Lactic acid | 1:1 | Clear liquid             |
| 42    | B/LA1        | 1,3-Butanediol | Levulinic acid | 1:1 | Clear liquid             |
| 43    | B/MA2        | 1,3-Butanediol | Malic acid | 1:1 | Clear liquid             |
| 44    | B/CA         | 1,3-Butanediol | Citric acid | 1:1 | Clear liquid             |
| 45    | B/GA2        | 1,3-Butanediol | Glycolic acid | 1:1 | Clear liquid             |
| 46    | B/GA1        | 1,3-Butanediol | Glutaric acid | 2:1 | Clear liquid             |
| 47    | P/LA2        | 1,2-Propanediol | Lactic acid | 1:1 | Clear liquid             |
| 48    | P/LA1        | 1,2-Propanediol | Levulinic acid | 1:1 | Clear liquid             |
| 49    | P/MA2        | 1,2-Propanediol | Malic acid | 1:1 | Clear liquid             |
| 50    | P/CA         | 1,2-Propanediol | Citric acid | 1:1 | Clear liquid             |
| 51    | P/GA2        | 1,2-Propanediol | Glycolic acid | 1:1 | Clear liquid             |
| 52    | P/GA1        | 1,2-Propanediol | Glutaric acid | 2:1 | Clear liquid             |
| 53    | X/LA2        | Xylitol | Lactic acid | 1:1 | Clear liquid             |
| 54    | X/LA1        | Xylitol | Levulinic acid | 1:1 | Clear liquid             |
| 55    | X/MA2        | Xylitol | Malic acid | 1:1 | Clear liquid             |
| 56    | X/CA         | Xylitol | Citric acid | 1:1 | Clear liquid             |
| 57    | X/GA2        | Xylitol | Glycolic acid | 1:1 | Clear liquid             |
| 58    | X/GA1        | Xylitol | Glutaric acid | 2:1 | Clear liquid             |
ride to form DESs with different alcohols and organic acids. Twenty-five combinations were investigated (Entries 16–40 in Table 1). Ten of them formed a stable clear liquid, 7 formed viscous liquids, and 8 could not form clear liquid. Lastly, some alcohols and organic acids that could easily form DESs with choline chloride or betaine were selected to form another 18 DESs (Entries 41–58 in Table 1), and all of them were found to be stable clear liquids. As a result, 50 different types of DESs were successfully produced (Entries 1–28, 35, 37, 39 and 40–58 in Table 1). After preparation, the DESs were dehydrated by incubating with 3 Å molecular sieves for several days before extraction experiments.

Selection of DESs

The produced 50 DESs were used as solvents to extract flavonoids from Ginkgo biloba leaves. At present, 70 % ethanol in water is the most efficient solvent for Ginkgo flavonoids extraction8. It has also been reported that the viscosity of DESs is generally high, which hinders the mass transfer of compounds from plant matrix to extraction solvent 18. Therefore, in order to compare the extraction effect with 70 % ethanol and reduce the viscosity of DESs, all produced DESs were mixed with deionized water at 7:3 (w/w) and used for the screening. The results are shown in Table 2. It could be found that all of the DESs could extract the Ginkgo flavonoids with varied extraction yields. Eighteen DESs (Entries 2, 3, 7, 8, 9, 10, 12, 17, 19, 23, 26, 34, 35, 37, 38, 39, 43 and 44 in Table 2) exhibited comparable extraction yields to the most efficient reference solvent, 70 % ethanol ($p$ >0.05). Three DESs (Entries 4, 11, and 40 in Table 2) gave obviously higher extraction yields than 70 % ethanol ($p$ <0.05). The extraction yields of ChCl/B, ChCl/LA1 and P/LA1 (Entries 4, 11 and 40 in Table 2) attained to 10.27, 10.32 and 10.30 mg g$^{-1}$, respectively, which were also the highest among the 50 DESs. One of the possible explanations for the high extraction yields of these three DESs may be the good liquidity, 

Table 2 – Amount of flavonoids extracted from Ginkgo biloba leaves using different DESs and reference solvents

| Entry | Solvents       | Extraction yield (mg g$^{-1}$) | Entry | Solvents       | Extraction yield (mg g$^{-1}$) |
|-------|----------------|-------------------------------|-------|----------------|-------------------------------|
| 1     | ChCl/G         | 9.05±0.31                     | 30    | PR/MA2         | 8.76±0.24                     |
| 2     | ChCl/EG        | 9.61±0.23                     | 31    | PR/CA          | 8.69±0.35                     |
| 3     | ChCl/P         | 9.77±0.08                     | 32    | PR/GA2         | 8.21±0.24                     |
| 4     | ChCl/B         | 10.27±0.11                    | 33    | B/LA2          | 8.73±0.16                     |
| 5     | ChCl/DS        | 9.17±0.15                     | 34    | B/LA1          | 10.08±0.09                    |
| 6     | ChCl/DG        | 7.77±0.07                     | 35    | B/MA2          | 10.04±0.13                    |
| 7     | ChCl/GA1       | 9.89±0.23                     | 36    | B/CA           | 8.45±0.24                     |
| 8     | ChCl/GA2       | 9.60±0.28                     | 37    | B/GA2          | 10.04±0.27                    |
| 9     | ChCl/MA1       | 9.58±0.16                     | 38    | B/GA1          | 9.55±0.27                     |
| 10    | ChCl/MA2       | 9.46±0.29                     | 39    | P/LA2          | 10.12±0.32                    |
| 11    | ChCl/LA1       | 10.32±0.14                    | 40    | P/MA2          | 6.17±0.10                     |
| 12    | ChCl/LA2       | 9.63±0.17                     | 41    | P/CA           | 7.57±0.25                     |
| 13    | ChCl/CA        | 8.67±0.11                     | 42    | P/GA2          | 10.14±0.19                    |
| 14    | ChCl/TA        | 8.67±0.09                     | 43    | P/GA1          | 10.16±0.18                    |
| 15    | ChCl/U         | 7.74±0.12                     | 44    | X/LA2          | 6.28±0.09                     |
| 16    | BE/G           | 9.14±0.22                     | 45    | X/MA2          | 7.30±0.12                     |
| 17    | BE/EG          | 9.51±0.18                     | 46    | X/CA           | 7.75±0.05                     |
| 18    | BE/P           | 8.40±0.26                     | 47    | X/GA1          | 7.44±0.09                     |
| 19    | BE/B           | 10.04±0.34                    | 48    | Water          | 8.93±0.32                     |
| 20    | BE/X           | 8.52±0.15                     | 49    | Ethanol        | 7.47±0.29                     |
| 21    | BE/DS          | 7.76±0.10                     | 50    | 1,3-Butanediol | 8.28±0.13                    |
| 22    | BE/GA2         | 8.28±0.25                     | 51    | 1,2-Propanediol | 8.24±0.24                |
| 23    | BE/MA1         | 9.84±0.31                     | 52    | Levulinic acid | 9.40±0.22                    |
| 24    | BE/OA          | 8.36±0.22                     | 53    | Lactic acid    | 8.20±0.19                     |
| 25    | BE/MA2         | 8.64±0.20                     | 54    | Choline chloride | 8.40±0.21           |
| 26    | BE/LA1         | 9.86±0.38                     | 55    |                 |                               |
| 27    | BE/LA2         | 8.72±0.27                     | 56    |                 |                               |
| 28    | BE/CA          | 8.61±0.12                     | 57    |                 |                               |

*Extraction conditions: 70 % (w/w) aqueous solution was used as solvent to extract Ginkgo flavonoids at 100 W and 60 °C for 60 min with a solid to solvent ratio of 1:15.
which was contributed by the liquid form of one or two constituents of DESs. The constituents of ChCl/B, ChCl/LA1 and P/LA1 (choline chloride, 1,3-butanediol, 1,2-propanediol or levulinic acid) were used to prepare 70 % aqueous solutions, and employed as solvents to extract the Ginkgo flavonoids. It could be found that all the constituent 70 % aqueous solutions gave lower extraction yields than the corresponding three constituent DES solutions (Entries 53–55, 57 in Table 2). These results showed that high extraction yields of ChCl/B, ChCl/LA1 and P/LA1 originated from DES formation. Compared with water as solvent, most of the DES solutions exhibited higher extraction yield (Entry 51, Table 2). ChCl/DG, BE/DS, P/MA2, and P/CA (Entries 6, 21, 41, 42 in Table 2) showed lower extraction yields, which may have resulted from their high viscosity. Besides viscosity, the extraction yield was also affected by other properties of DESs, which could be adjusted by the nature of constituent. Fixing one component of DES, the extraction yield of a different DES was attempted to be correlated with the choice of another component, but the correlation failed. As for the DESs, their properties are complicated because DESs are in fact binary mixtures, in contrast to the protic organic solvents which are pure substances. This highlights the difficulty in predicting the extraction yield of a DES. Finally, ChCl/B, ChCl/LA1 and P/LA1 were selected for further investigation.

### Comparison of different extraction methods

Ultrasound-assisted extraction (UAE) was employed as the extraction method in the initial screening of DESs due to its simplicity for comparing multiple samples at the same time. Based on previous reports involving DES-based extraction, three other commonly used extraction methods, stirring, heating, and heating + stirring, were compared with UAE method with ChCl/B, ChCl/LA1 and P/LA1 being used as the extraction solvents (Table 3).

At 25 °C, the stirring and ultrasonic method exhibited no obvious differences in extraction yields for the three DESs. At 60 °C, however, heating and heating + stirring method showed significant differences in extraction yields for the three DESs. The extraction yield of heating + stirring method was almost two times higher than those of heating method for the three DESs. While stirring at 150 rpm, the temperature increased from 25 °C to 60 °C, which enhanced the extraction yields for the three DESs by about 40 %. Stirring or heating alone was inefficient, and combination of heating and stirring should be a good choice. As far as the three DESs are concerned, the extraction yield of ChCl/B was always lower than that of ChCl/LA1 and P/LA1. Accordingly, extraction conditions were further optimized based on stirring method at 25 °C using ChCl/LA1 and P/LA1 as the extraction solvents.

### Optimization of the extraction conditions

**Effect of water content in DES**

The addition of water to DES can cause a decrease in DES viscosity, which is beneficial to the mass transport from plant matrices to solution. Also, addition of water to DES can modulate the polarity of the DES, which may preferably match the polarity of the target compounds and give better extraction yield. In order to determine the optimum water content in DES for Ginkgo flavonoids extraction, the extraction procedures were performed in ChCl/LA1 and P/LA1 with different water content (0–100 %, w/w). The results are shown in Fig. 1. It could be observed that the extraction yields reached maximum when the water content was 40–60 % (w/w) for both of the DESs. When the water content increased from 0 to 40 % (w/w), the extraction yields increased obviously. However, high-

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**Table 3 – Amount of flavonoids extracted from Ginkgo biloba leaves using different extraction methods**

| DESs  | Heating | Heating + Stirring | Stirring | Ultrasound |
|-------|---------|--------------------|----------|------------|
| ChCl/B | 4.44±0.21 | 10.01±0.19 | 5.66±0.17 | 4.34±0.18 |
| ChCl/LA1 | 5.41±0.08 | 10.27±0.16 | 6.61±0.16 | 6.22±0.04 |
| P/LA1 | 5.55±0.05 | 10.24±0.16 | 6.47±0.19 | 6.09±0.04 |

*Extraction conditions: 70 % (w/w) DES aqueous solution was used as solvent to extract Ginkgo flavonoids at a solid to solvent ratio of 1:15 with heating method (at 60 °C and 0 rpm), stirring method (at 25 °C and 150 rpm), heating + stirring method (at 60 °C and 150 rpm), and ultrasonic method (at 50 W and 25 °C).*

**Fig. 1 – Effect of the water content in DES-water mixture on the extraction yield of Ginkgo flavonoids. Varied DES aqueous solution was used as extraction solvent at a solvent to solid ratio of 15:1 with stirring at 25 °C and 150 rpm for 10 min.**
er concentration of water in ChCl/LA1 and P/LA1 (e.g. 80 %, w/w) led to the decrease in the amount of target flavonoids extracted. The addition of water could effectively reduce the viscosity, and could favorably affect the solvent polarity, while excessive water content would probably decrease the interactions between DES and flavonoids, and further increase unfavorably the polarity of the solvent mixture. The concentrations of 40 % and 60 % (w/w) water in ChCl/LA1 and P/LA1 were selected for subsequent experiments.

**Effect of extraction temperature**

Extraction temperature is a crucial factor benefitting the increase of extraction yield according to the results of the comparison of different extraction methods. The Ginkgo flavonoid glycosides are adsorbed on the plant matrix by physical adsorption and/or chemical interactions. Raising temperature is one of the most convenient measures for decreasing the adsorption and/or interactions for desorption and dissolution of the flavonoids compounds to the extraction solvent. Also, the DES viscosity will decrease and its diffusivity will increase at higher temperature, which will improve the release of Ginkgo flavonoid compounds from plant matrix to the DES. The effect of temperature on the extraction yield of Ginkgo flavonoids was investigated at the temperatures of 25, 30, 35, 40, 45, 50, 55 and 60 °C, respectively. The results are shown in Fig. 2. It could be found that the extraction yields increased by raising temperature from 25 °C, and reached maximum at about 50 °C for both ChCl/LA1 and P/LA1 containing 40 % (w/w) water. This result could be explained by the increase in mass transport and decrease in viscosity with the increase in temperature as mentioned above. However, as for ChCl/LA1 and P/LA1 containing 60 % (w/w) water, the temperature increase could not improve the extraction yields remarkably. Addition of 60 % (w/w) water might adversely affect the hydrogen bonding between the two components of DES, and influence the van der Waals force between DES and flavonoids, which would result in insignificant improvement of extraction yield. The ChCl/LA1 containing 40 % (w/w) water produced higher extraction yield than that of P/LA1 containing 40 % (w/w) water, which might be due to a better polarity match of ChCl/LA1 containing 40 % (w/w) water with target Ginkgo flavonoid glycosides, and/or due to a more pronounced viscosity decrease of ChCl/LA1 containing 40 % (w/w). Thus, ChCl/LA1 containing 40 % (w/w) was selected for further study.

**Effect of the DES-water mixture to solid ratio**

A low ratio of solvent to solid may lead to an incomplete extraction, but a high ratio of solvent to solid can make the process complex and lead to DES waste. The effect of varied ratios between DES volume and Ginkgo biloba leaves powder weight (7.5:1, 10:1, 12.5:1, 15:1, 20:1, 30:1, and 50:1) on the extraction yield of Ginkgo flavonoids was investigated. As shown in Fig. 3, the extraction yield of the target Ginkgo flavonoids increased a little with the increase in DES volume before DES-water mixture to solid ratio reached 10:1, and then the extraction yield remained almost unchanged with a further increase in DES volume. At DES-water mixture to solid ratio of 10:1, the Ginkgo biloba leaves powder and DES-water mixture might have mixed thoroughly. Hence, further increase in solvent to solid ratio could not lead to increase in extraction yield of Ginkgo flavonoids. Considering the DES consumption, DES-water mixture to solid ratio of 10:1 was used for the extraction of target flavonoids.
Effect of extraction time

The choice of an appropriate extraction time is important to ensure the extraction equilibrium of target Ginkgo flavonoids between Ginkgo biloba leaves powder and DES-water mixture. The extraction was carried out with different extraction times ranging from 5 to 40 min. The results are shown in Fig. 4. The extraction yield of Ginkgo flavonoids increased slowly with the extension of extraction time within the first 15 min. After 15 min, the extraction time had little effect on the extraction yield, indicating that Ginkgo flavonoids reached their extraction equilibrium at around 15 min. The extraction yield at 15 min was 10.32±0.01 mg g⁻¹. The sample extracted for 15 min was centrifuged at 10000 rpm for 10 min, and the precipitate was re-extracted using the same method for another two times. The result showed that the extraction yield was about 0.013 mg g⁻¹ in the second extraction, and could not be determined in the third extraction. The adsorption yields for HPD450, HPD417, DM130 and ADS-17 were all lower than 60 %. The adsorption yield for D101 reached 85.2 %. The AB-8 resin gave the maximum adsorption yield of 93.7 %. The AB-8 resin adsorbing Ginkgo flavonoids washed twice with 95 % (v/v) ethanol solution could free almost all the Ginkgo flavonoids to the ethanol solution. Therefore, the AB-8 resin was effective for recovering the target Ginkgo flavonoids.

Conclusion

This work has demonstrated that ChCl/LA1 containing 40 % (w/w) water can be used as an alternative solvent to efficiently extract Ginkgo flavonoids from Ginkgo biloba leaves. This DES-based method gives a very high extraction efficiency of 99.87 % in only 15 min. The Ginkgo flavonoids in the DES extraction solution can also be easily recovered by the AB-8 macroporous resin.

ACKNOWLEDGEMENTS

This work was supported by the “China Postdoctoral Science Foundation (2016M600417 and 2017T100373)”, the “333 project of Jiangsu Province (BRA2017458)”, the “Six Talent Peaks Project in Jiangsu Province (2015-JY-016)”, the “Open Project of State Key Laboratory of Natural Medicines (No. SKLMKF201802)”, and “A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, PAPD”.

Recovery of Ginkgo flavonoids from the DES extraction solution

The recovery of target compounds from DES extraction solution is a challenging task due to the negligible vapor pressure and the generally high water miscibility of DESs. Several approaches have been suggested to recover the target compounds, including the application of antisolvents, recrystallization, back extraction, chromatographic techniques, and countercurrent separation. In this work, the recovery of the target Ginkgo flavonoids from DES extraction solution was attempted using six macroporous resins. The results showed that all the six macroporous resins could adsorb the Ginkgo flavonoids with different adsorption yield. The adsorption yields for HPD450, HPD417, DM130 and ADS-17 were all lower than 60 %. The adsorption yield for D101 reached 85.2 %. The AB-8 resin gave the maximum adsorption yield of 93.7 %. The AB-8 resin adsorbing Ginkgo flavonoids washed twice with 95 % (v/v) ethanol solution could free almost all the Ginkgo flavonoids to the ethanol solution. Therefore, the AB-8 resin was effective for recovering the target Ginkgo flavonoids.
**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| DESs         | Deep eutectic solvents |
| HPLC         | High performance liquid chromatography |
| SFE          | Supercritical CO₂ extraction technology |
| HBDs         | Hydrogen bond donors |
| HBAs         | Hydrogen bond acceptors |
| UAE          | Ultrasound-assisted extraction |
| HPD450, HPD417 | Different macroporous resins |
| DM130, ADS-17 |                         |
| AB-8, D101    |                         |

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