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Antisolvent precipitation for the preparation of high polymeric procyanidin nanoparticles under ultrasonication and evaluation of their antioxidant activity *in vitro*

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ABSTRACT

An improved method of ultrasonic antisolvent precipitation was used to prepare micronized high polymeric procyanidins (HPC). Response surface methodology (Plackett–Burman and Box–Behnken design) was employed to predict the optimal preparation conditions and satisfactory mean particle size. Among seven parameters, three parameters (i.e., ultrasonic irradiation power, ultrasonic-stirring time, and stirring speed) were identified as the most significant variables using Plackett–Burman design; thus, these three parameters were further optimized using Box–Behnken design. The optimal preparation conditions for micronized HPC were obtained as follows: dropping speed of 4 mL/min, HPC solution concentration of 0.3 mg/mL, ratio of antisolvent and solvent of 5 mL/mL, precipitation temperature of 10 °C, ultrasonic-stirring time of 14 min, ultrasonic irradiation power of 620 W, and stirring speed of 760 r/min. A minimum mean particle size of 96 ± 2 nm was achieved under the aforementioned conditions. The obtained micronized HPC was analysed by scanning electron microscopy, Fourier transform infrared spectroscopy, thermogravimetric and X-ray powder diffraction patterns. Micronized HPC enjoyed the higher quantity dissolved and exhibited stronger antioxidant activity in compared to the unprocessed HPC. These results demonstrated that the improved method has great potential for the production of micronized particles.

1. Introduction

Decreasing particle size is an acceptable, effective way to enhance drug bioavailability. Several techniques have been applied to fabricate micronized drug particles, such as traditional antisolvent precipitation [1,2], supercritical antisolvent precipitation [3–5], emulsification [6,7], spray drying [8,9], and milling methods [10]. Among these techniques, antisolvent precipitation is the most convenient, effective and lowest-cost method to prepare drug nanoparticles. In this method, two miscible solvents (i.e., solvent and antisolvent) were applied to fabricate nanoparticles as follows: complete dissolution of crude drug samples in the solvent followed by addition of antisolvent to the drug solution to generate nanoparticles by fast desolvation. Notably, the two mixing methods are associated with antisolvent precipitation as reported in previous studies: mixing by adding antisolvent to solvent has been used to prepare ursolic acid nanoparticles and curcumin nanosuspensions [11,12]; and mixing by adding solvent to antisolvent have also been used to fabricate licorice extract microparticles and micronized ellagic acid [13,14].

Ultrasound has been employed in antisolvent crystallization techniques; it can facilitate the nucleation process and microparticle fabrication with a narrow particle size distribution [15–17]. A reasonable principle involves cavitation triggered by ultrasonication. When ultrasound waves penetrate the saturated solution, the formation and disruption of cavitation bubbles can give rise to high heating and cooling velocity. In addition, high temperature and pressure were generated in the cavitation bubbles, which could promote effective collision of molecules and excessive partial over saturation, leading to the primary nucleation [17]. Additionally, previous research has indicated the disruption of cavitation bubbles by high-speed micro-jets, which can decrease the particle size of large crystals [18]. To the best of our knowledge, there is no report on the preparation of nanoparticles with ultrasonication-assisted antisolvent precipitation. Therefore, we sought to combine ultrasonic waves with shearing and homogeneous processes to control the morphologies and sizes of nanoparticles.

Procyanidins, belonging to the subclass of proanthocyanidins, primarily consist of monomeric, oligomeric, and polymeric structures of (+)-catechin and (−)-epicatechin units [19]. Their structural diversity

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is highly dependent on interflavanoid junction and the type and amount of flavan-3-ol units [(C6-C3-C6)n] [20]. The mean degree of polymerization (mDP) is generally employed to investigate the size of procyanidins. Oligomeric procyanidins (OPC) are those with an mDP of 2–6, while the mDP of highly polymeric procyanidins (HPC) is above 6 [21]. Procyanidins are naturally indwelling polymeric phenolic compounds, which are abundant in plant fruits, seed, leaves, and bark [4,22–24]. Procyanidins have attracted considerable attention in the pharmaceutical and food chemistry fields due to their correlative biological characteristics and beneficial pharmacological effects on human diseases, such as dysarteriotony [25], angiocardiopathy [26], diabetes [27,28], and certain types of cancer [29]. Nonetheless, previous reports have suggested HPC are not as easily absorbable as oligomers in the gastrointestinal tract [30]. Thus, the development of a promising method to enhance the bioavailability of procyanidins, especially the predominance of polymers, would be significant.

Cinnamomum longepaniculatum (Gamble) N. Chao, a member of the Lauraceae family, is primarily distributed in southwestern China [31]. *C. longepaniculatum* leaves are abundant in high value-added essential oil, which is a major source of eucalyptol, and *C. longepaniculatum* therefore is widely cultivated in Yibin city (Sichuan province) as the major economic crops [32]. In recent years, several studies have indicated that *C. longepaniculatum* leaves are also rich in flavonoids [33] and procyanidins [34].

The present study seeks enhance the antioxidant activities of *C. longepaniculatum* HPC by decreasing their particle size using an improved technique of ultrasonically assisted antisolvent precipitation (UASP). First, seven variables that can affect the mean particle size (MPS) of micronized HPC, including dropping speed, ultrasonic-stirring time, stirring speed, ratio of antisolvent and solvent, ultrasonic irradiation power, HPC solution concentration, and precipitation temperature, were sieved by Plackett–Burman design; these were further optimized with the Box–Behnken design for the methodology of response surface. Additionally, processed and unprocessed HPC samples were characterized through scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric (TG) and X-ray powder diffraction patterns (XRD). The percentage dissolved value and antioxidant activity of the processed and unprocessed HPC were also investigated.

2. Materials and methods

2.1. Chemicals and materials

The chemicals 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), and oligomeric proanthocyanidins were purchased from Sigma Aldrich (St, Mo, USA). Other analytical-grade applied chemicals were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). A Milli-Q water cleansing apparatus (Millipore, MA, USA) was used to prepare the water used in the experiments.

C. longepaniculatum leaves were collected in November 2015 from Yibin city (Sichuan, China) and authenticated by Prof. Kailin Mo from the Sichuan Academy of Forestry, China. The leaves were dried at room temperature, powdered by a pulverizer and sieved to a 40–60 mesh size.

2.2. HPC extraction and determination of mDP

Leaf powders (1 kg) were defatted three times (4 h each) with 10 L petroleum ether (boiling range 30–60 °C) using a reflux extraction method. The de-oiled *C. longepaniculatum* leaf powder (900 g) was extracted using a hot reflux extraction method at 85 °C with 9-L ethanol solutions (80%, v/v) for 4 h. Crude extracts were evaporated under vacuum at 65 °C to a small volume (approximately 1 L) and filtered. Filter cakes were washed with a small amount of deionized water, and 500 mL of deionized water was added before the cakes were subjected to lyophilization to obtain the HPC-rich fraction. The filter liquor was extracted six times with 2000 mL ethyl acetate followed by recycling of ethyl acetate with a vacuum rotary evaporator. Next, 500 mL deionized water was added to dissolve the evaporation residues, which were finally subjected to freeze-drying to obtain the OPC-rich fraction. After the above steps, 15.52 g of the OPC-rich fraction and 22.43 g of the HPC-rich fraction were collected. OPC and HPC samples were analysed using phloroglucinolysis according to the literature [35]. The mDP was calculated as described in a previous study [36] as 12.09 for HPC and 2.34 for OPC.

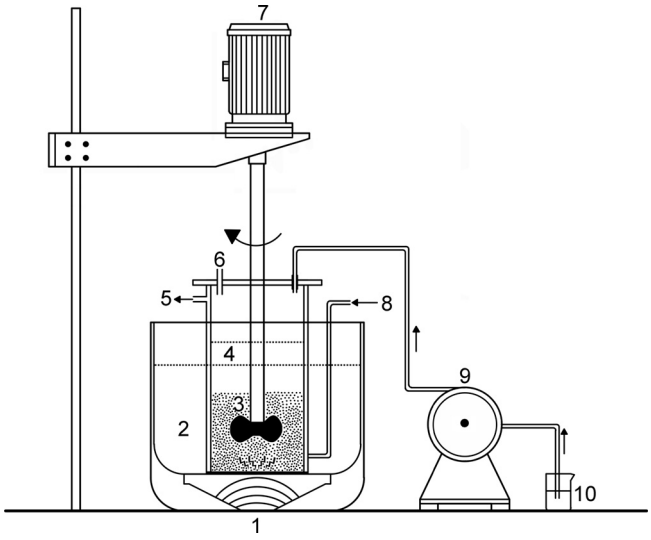

Fig. 1. The apparatus of UASP for the preparation of processed HPC. 1 ultrasonic generator; 2 ultrasonic bath; 3 shear head; 4 antisolvent; 5 freezing brine out; 6 blowhole; 7 electromotor; 8 freezing brine in; 9 peristaltic pump; 10 HPC solution.

2.3. Apparatus of UASP method

The UASP apparatus diagram is presented in Fig. 1. A KH 700DE ultrasonic bath (Kunshan, Jiangsu, China) and an adjustable agitator (Ningbo Xinzhi Biotechnology Co., Ltd, Zhejiang, China) were employed for UASP process. A round bottom flask (maximum volume 25 mL) was employed for the preparation of HPC microparticles and the total volume of solvent and antisolvent was 18 mL in the system. A total volume of 6 L distilled water was filled in its inner groove (interior size 50 × 30 × 15 cm) during UASP process. The ground bottom flask (maximum volume 25 mL) contained antisovent was placed in the ultrasonic bath for the UASP process. As the results obtained from the preliminary experiments, an ultrasonic power higher than 400 W facilitates to prepare nanosized HPC particles and thus the power range over 400 W was selected for the preparation of nanosized HPC particles. In this study, nominal ultrasonic power instead of actual ultrasonic power was selected to show input power in the UASP procedure. Based on the literature [37], many factors can affect acoustic intensity in ultrasound field, and therefore it was inferred that the actual ultrasonic power acted on the ground bottom flask was lower than the 400 or 800 W of nominal ultrasonic power. HPC solution (10) was injected by a peristaltic pump (9) from the top of the flask. Especially, the ultrasonic bath equipped with six ultrasonic probes was used in the UASP apparatus. The UASP system temperature was controlled by circulation brine water as shown in the Fig. 1. Stirring speed, ultrasonic irradiation power and time are adjustable through the feedback/control during experiments to obtain the requisite operating parameters. The freezing brine in and freezing brine out in the jacket reactor are connected to the