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Optimisation of pressurised liquid extraction of antioxidants from black bamboo leaves

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ABSTRACT

To develop an efficient green extraction approach for recovering bioactive compounds from natural plants, the potential of using pressurised liquid extraction (PLE) was examined on black bamboo (*Phyllostachys nigra*) leaves, with ethanol/water as solvents. The superheated PLE process showed a higher recovery of most constituents and antioxidative activity, compared to reflux extraction, with a significantly improved recovery of the total phenolic (TP) and flavonoid (TF) content and DPPH radical scavenging ability. For a broad range of ethanol aqueous solutions and temperatures, 50% EtOH and 200 °C (static time: 25 min) gave the best performance, in terms of the TP and TF (75% EtOH) content yield and DPPH scavenging ability (25% EtOH). Under the optimised extraction conditions, eight main antioxidative compounds were isolated and identified with HPLC-ABTS$^+$ assay guidance and assessed for radical scavenging activity. The superheated extraction process for black bamboo leaves enhanced the antioxidant properties by increasing the extraction of the phenolic components.

1. Introduction

A high level of fruit and vegetable consumption has shown the association of phenolic compounds with health benefits (Parr & Bolwell, 2000). The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim, Tagliaferro, & Bobilya, 2002). Phenolic compounds could be a major determinant of the antioxidant potential of foods (Parr & Bolwell, 2000), and therefore could be a natural source of antioxidants.

It has become increasingly important to obtain antioxidants from common edible plant materials. In Korea, local inhabitants enjoy black bamboo teas made from roasting black bamboo leaves. The roasting process enhances the antioxidative activity as well as the flavours and taste (Kim, Jeon, Kang, Kim, Lee, & Um, 2012). Bamboo leaves have been used as a Chinese medicinal material for reducing the energy of "fire" (an element usually related to inflammation) and to treat cardiovascular disease, arteriosclerosis and hypertension (Yuan, 1983). In a previous study, we reported that black bamboo (*Phyllostachys nigra*) leaf extracts and the isolated phenol compounds were effective at inhibiting aldose reductase and advanced glycation endproducts *in vitro* (Jung et al., 2007) and protecting against oxidative stress-induced retinal ganglion cells death (Lee et al., 2010).

The search for plant-derived biomaterials has stimulated research interest in using more efficient methods for extracting polyphenolic compounds. The pressurised liquid extraction (PLE) method is an important sample preparation technique for extracting bioactive compounds. This technique is a green extract method due to its decreased solvent use, short operating time and light- and oxygen-free environment (Acar, Gokmen, Pellegrini, & Fogliano, 2009). This technique causes desirable and/or undesirable changes in the physical, chemical and nutritional properties of the extracted materials due to the control experiment conditions (Rostagno, Palma & Barroso, 2004; José, Pedro, & María, 2006). One of the main desired outcomes of the PLE process is the increase in antioxidant activity, which occurs mainly due to the formation of Maillard reaction products (MRPs). The PLE process has been used to increase the extraction efficiency of various bioactive compounds (Shang et al., 2010; José et al., 2006).

In this study, the optimised conditions for extracting black bamboo leaves under a superheated PLE process were investigated for total polyphenol and flavonoid content as well as the DPPH radical scavenging ability. The newly produced and increased antioxidative compounds at the optimised extraction condition were identified and assessed for antioxidative activity.

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2. Materials and methods

2.1. Chemicals and plant materials

All HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). The organic solvents used for extraction were purchased from Daejung (Gyonggi, Korea). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH•), ascorbic acid, Folin–Ciocalteu's phenol reagent, catechin and potassium persulfate were purchased from Sigma–Aldrich Chemicals (Saint Louis, MO, USA). Deuterium solvent methanol-*d*4 was purchased from Cambridge Isotope Laboratory (USA). Fresh leaves (500 g) of *P. nigra* Munro were harvested on September 12, 2010, in Gangnueng City (Gangwon-do, Korea), and a voucher specimen (No. BBL-0003) was deposited at the Herbarium of KIST, Korea. The leaves were air-dried in the shade and kept at room temperature until use. Column chromatography was performed over silica gel 60 (Merck, particle size 230–400 mesh).

2.2. PLE procedure

PLE was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA). Samples were loaded into the stainless steel cell (11 mL) with sea sand (particle size 30–50 mesh, Fisher Chemicals) above the sample to avoid any void spaces. Various temperature ranges, ethanol:water concentrations, sample sizes, and extraction times were used to identify the optimal conditions for extracting polyphenols and investigating the antioxidative activity. A standard stepwise PLE extraction protocol was used for all extractions. (1) The extraction cell was placed in the carrousel and heated up to the temperature specified by the univariate design (A °C). (2) The cell was filled with the specified ethanol and water concentration (B%) until a pressure of 1500 psi was reached. (3) Static extraction (C min) when the pressure and temperature were reached was then maintained. (4) The cell was rinsed with an additional 40% of the volume of the solvent mixture that had already crossed the cell. Extracts were collected in 60 mL glass vials. The volume was made up to 25 mL with extraction solvent and filtered through a 0.45 μm membrane filter (Agilent Technologies, Santa Clara, CA, USA) before the total phenolic and flavonoid and DPPH radical scavenging analysis and injection into the HPLC system.

2.3. Polyphenol assays

2.3.1. Determination of total phenolic content

The total phenolic content of the extracts was determined with the Folin–Ciocalteu colorimetric method, with some modification. Briefly, black bamboo leaf extract supernatant was diluted twice. Each sample solution (2 μL) was mixed with 78 μl water and Folin–Ciocalteu's reagent 20 μL for 5 min. Then sodium carbonate solution (100 μl, 20%, v/v) was added. The mixture stood for 30 min in the dark at room temperature and was measured at 730 nm with the Synergy HT-Multi-microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The final result was expressed as milligrams of catechin equivalents (CE) per 100 g dry sample of black bamboo leaves.

2.3.2. Determination of total flavonoid content

The total flavonoid content was measured using a colorimetric method, with modification. For this, 2% $AlCl_3(H_2O)$ 100 mL was prepared, and a 10 μL sample was taken from the extraction solution, which had been diluted twice and mixed with 90 μl ethanol and 100 μl $AlCl_3(H_2O)$ for 5 min. The absorbance was read at 430 nm with a Synergy HT-Multi-microplate reader in triplicate. The final result was expressed as the milligram of quercetin equivalents per 100 g dry sample of black bamboo leaves.

2.4. Measurement of in vitro antioxidative activity

2.4.1. DPPH assay

A modified version of the diphenyl picrylhydrazyl (DPPH) assay was used to measure the *in vitro* antioxidant activity, using ascorbic acid as the standard (Goupy, Hugues, Boivin, & Amiot, 1999). Standard samples were prepared by diluting an ethanolic ascorbic acid stock solution (0.1 mg/mL). The ascorbic acid standard and blanks were used for the calibration curve. The results are expressed in mg ascorbic acid/100 g dry weight (mg AA/100 g DW). A working DPPH solution (0.15 mg/mL) was prepared by dissolving 15 mg DPPH in 100 mL methanol. Before analysis, all extracts were diluted twice from the 25 mL original extracts. A serial dilution of the extracts (100 μL) was prepared and added to 100 μL of the DPPH working solution in a 96 well-plate. The 96 well-plates were left in the dark for 30 min at room temperature. The absorbance was then measured against ethanol at 515 nm with a Synergy HT Multimicroplate reader (Bio-Tek Instruments). The decrease in the absorbance of a sample was calculated compared to a blank sample and corrected for the absorbance of the sample extract itself. The relative decrease in absorbance (PI) was then calculated as follows: PI (%) = $1 - (A_s/A_b)$, where A_s was the absorbance of sample extract and A_b the absorbance of blank. The antioxidative activity was defined as the concentration of the sample extract necessary to scavenge 50% of the DPPH radicals (SC_{50}). For the samples, the SC_{50} unit is the μl of extracts. In all experiments, the SC_{50} of ascorbic acid (μg/mL) was determined as well. The final results for the antioxidative activity were determined using the following equation: antioxidant activity = $(SC_{50}/SC_{Sample})/4$. The antioxidative activity was expressed in g ascorbic acid equivalent (AAE) per 100 g dry weight sample (g AAE/100 g DW).

2.4.2. Antioxidant activity determination with on-line HPLC-ABTS$^+$ screening system

To investigate the antioxidant activity profile of the black bamboo extracts, the on-line HPLC-ABTS$^+$ screening system was applied. The Agilent 1200 analysis HPLC system (Agilent Technologies, Santa Clara, CA, USA) was fitted with an additional pump to supply the ABTS radical solution. The ABTS solution was prepared with the same method as that used for off-line. An aliquot of 10 μl black bamboo extract methanol solution (10 mg/mL) (40 °C and 200 °C with PLE) was injected into the on-line HPLC-ABTS$^+$ system.

The individual compound was separated with an YMC-PACK C18 analytical column (150 × 4.6 mm i.d., 3 μm particle size, YMC, American, Inc.). The HPLC condition was expressed as follows: The mobile phase was acetonitrile (solvent A) and water (solvent B), a gradient system of solvent A from 10% to 64% in 40 min with flow rate 1 mL/min was used. The ABTS solution was supplied with a flow rate of 0.5 ml/min. The chromatogram was recorded at 210 nm and 280 nm as the positive peaks, and the visible detector was set at 734 nm to measure the decrease in the ABTS radicals as the negative peak (Fig. 1).

2.5. Experimental design for optimisation

The univariate method was used to optimise polyphenol and antioxidative activity extraction from black bamboo leaves. The sample surface area affects the interaction between the sample and solvent; therefore, various sample sizes (A: 425 μm, 850 μm, 2 mm, 4.75 mm, 6 mm) were tested. Since an increase in temperature can improve the extraction of natural compounds, a series of experiments at different temperatures (B: 40, 80, 120, 160, 200 °C) was performed to determine the best extraction tempera-