Efficient Extraction of Caffeic Acid Derivatives from Adventitious Roots of *Echinacea purpurea*

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Abstract

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Echinacea species are popular herbal medicine and food supplements for enhancing the immune system. This study was conducted with the aim of developing an efficient heat reflux extraction of caffeic acid derivatives from dried adventitious roots of *Echinacea purpurea* obtained in bioreactor cultures. Water, methanol (20, 40, 60, 80, and 100%), and ethanol (20, 40, 60, 80, and 100%) were used as solvents for the extraction of caffeic acid derivatives. Another parameter used for the optimisation was the solvent temperature during extraction. The treatment of samples with 60% ethanol at 60°C for 2 h proved to be the most suitable procedure. This treatment was also responsible for the higher yields of total phenolics, flavonoids, and polysaccharides.

Keywords: adventitious roots; caftaric acid; chlorogenic acid; chichoric acid; *Echinacea purpurea*

Echinacea purpurea (L.) Moench is a traditional North American perennial medicinal herb that has gained international popularity because of its nutraceutical and medicinal properties (Li 1998). The extracts from the plant have shown antioxidative, antibacterial, antiviral, and antifungal properties and are used for treating common cold and respiratory and urinary diseases (Barrett 2003). In admixture, the roots of this species are traditionally used in dietary supplements and herbal medicines. The most important potential active compounds in *E. purpurea* are caffeic acid derivatives, namely caftaric acid, chlorogenic acid, and chichoric acid. Of the caffeic acid derivatives, chichoric acid has shown immunostimulatory properties, promoting phagocyte activity *in vitro* and *in vivo*. In recent years, plant organ cultures have become an alternative to whole plants for the production of valuable bioactive compounds. Adventitious roots have been induced in *E. purpurea* and cultivated in large-scale bioreactors for the production of biomass which can meet the demands of pharmaceutical, nutraceutical, and herbal industry (Wu *et al*. 2007a, b).

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The extraction is the first important step for the recovery of the bioactive compounds from the plant raw materials. The extraction technologies must be versatile, relatively simple, and safe for both the operating personnel and the consumers and inexpensive to use. These characteristics apply to conventional procedures such as steam distillation and solvent extraction. The solvent extraction of plant materials is mostly based on the correct choice of solvents and the use of heat and/or agitation to increase the solubility of materials and the rate of the mass transfer (Wang & Weller 2006).

The objective of the present work was to establish the solvent extraction protocol for the isolation of caffeic acid derivatives from the dried adventitious roots of *E. purpurea*. Different solvents (water, methanol, and ethanol) and different extraction temperatures were evaluated for the solvent extraction of caffeic acid derivatives.

MATERIAL AND METHODS

Adventitious root samples and extraction solvents. Adventitious roots which were induced from the natural roots of *Echinacea purpurea* were cultivated in suspension cultures (5 l capacity 5 l airlift bioreactors) by following the method of Wu *et al*. (2007a). The adventitious roots were harvested from the bioreactor cultures and were dried at 40°C in a forced air oven to the moisture content of about 10%. After drying, the roots were stored at –20°C and protected from light and humidity until required for the analysis. The dried roots were ground in an IKA M20 grinder (Staufen, Germany) immediately prior to the extraction. Acetonitrile, ethanol, and methanol were of HPLC grade (Fisher Scientific, USA). Water was purified by Millipore Q system (Millipore, Bedford, USA).

Extraction of caffeic acid derivatives. The extraction was carried out in 100 ml round bottom flask fitted with a cooling condenser. The doses of 2 g of powdered roots were put into round bottom flasks and the extraction was carried at 80 ± 1°C for 1 h by using 40 ml of the following solvents: distilled water, methanol (20, 40, 60, 80 and 100%) and ethanol (20, 40, 60, 80, and 100%) to find out the suitable solvent for the extraction of caffeic acid derivatives. In another set of experiments, the samples (2 g of dried powdered roots) were extracted by using 40 ml of 60% ethanol and the extraction was carried out at 40, 60, and 80°C for 1 h to select the suitable temperature for the extraction. In both experiments, the cooled extract was filtered through double layers of Whatman No. 1 filter paper. The residue was recovered and re-extracted once more using fresh solvent under the same conditions as mentioned above. The condenser was washed with 20 ml of the solvent and the washings were added to the extract. The combined extract (100 ml) was used for the quantification of caffeic acid derivatives, phenolics, flavonoids, and polysaccharides.

Determination of total phenolic contents. The content of total phenolics in the plant methanolic extracts was analysed spectrophotometrically using a modification of Folin-Ciocalteu colorimetric method according to the method of Wu *et al*. (2007b). 100 µl of methanolic extracts were mixed with 2.5 ml deionised water, followed by the addition of 0.1 ml (2N) Folin-Ciocalteu reagent. The mixtures were well stirred and allowed to stand for 6 min before 0.5 ml of a 20% sodium carbonate solution was added. The colour developed after 30 min at room temperature and the absorbance was measured at 760 nm using a UV visible spectrophotometer (UV-1650PC, Shimadzu, Japan). The measurement was compared to a standard curve of gallic acid solution prepared and expressed as mean mg of gallic acid equivalent per gram of the plant material dry weight (DW) for the triplicate extracts.

Determination of flavonoid contents. Total flavonoid content was determined by a colorimetric method (Zhishen *et al*. 1999). Briefly, 0.25 ml of methanolic plant extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by the addition of 0.75 ml of 5% sodium nitrite solution. After 6 min, 0.15 ml of 10% aluminum chloride solution was added and the mixture was made up to 2.5 ml with distilled water and well mixed. The absorbance was measured immediately at 510 nm using a spectrophotometer (UV-1650PC, Shimadzu). The results were expressed as mean mg of (+)-catechin equivalents per gram of the plant material dry weight for the triplicate extracts.

Determination of polysaccharide content. After the extraction, the sediment was collected and desiccated in an oven at 60°C. 0.2 g of the sediment was resuspended in 5 ml of 5% sulphuric acid and placed in boiling water for 2 hours. After acidic hydrolysis, the liquid-solid mixture was diluted to 50 ml with distilled water. The supernatant was separated by sedimentation, and the polysaccharide in the supernatant was assayed according to the

carbazole reaction method as follows. A sample of 0.2 ml taken from the above supernatant was mixed with 6 ml concentrated sulphuric acid, held in a boiling water bath for 20, min and cooled. Then, 0.2 ml carbazole-absolute ethanol (0–15% v/v) was added and the contents vigorously were mixed. After the reaction time of 2 h in darkness at room temperature, the purplish red colour developed and the absorbance was measured at 530 nm. β-Galacturonic acid (0. 50, 100, 200, 400, and 600 mg/ml) was used as the standard.

Determination of content of caffeic acid derivatives. HPLC was used to analyse the caffeic acid derivatives. The HPLC peaks were identified by comparing their retention times with those of the standard samples and extraction, which were determined under the same chromatographic conditions. The extraction and analysis of caffeic acid derivatives were done by the method of Pellati *et al*. (2004). The caffeic acid fractions were analysed using an HPLC system with XTerra® RP 18 column (particle size 3.0 µm, 150 mm × 3 mm). The mobile phase was (A) water and (B) acetonitrile. The gradient elution was modified as follows: initial 10% B for 40 min; 25% B for the next 11 min; 50% B for 1 min; recycling to the initial condition for 8 min with a flow rate of 0.3 ml/min. Caffeic acid derivatives were detected at 330 nm. The standards were obtained from Chroma Dex (Laguna Hills, CA, USA).

Statistical analysis. Statistical analysis involved the use of the Statistical analysis systems software package. The analysis of variance was performed by ANOVA procedures. Significant differences between the means were determined by Duncan's multiple range test.

RESULTS AND DICUSSION

Comparison of different solvents for the extraction of caffeic acid derivatives

Different solvents will yield different extracts and extract compositions (Zarnowski & Suzuki 2004); therefore, a suitable extracting solvent should be selected for the extraction of nutraceuticals (Wang & Weeler 2006). Distilled water, methanol (20, 30, 60, 80, and 100%) and ethanol (20, 30, 60, 80, and 100%) were used as solvents for the extraction of caffeic acid derivatives from the dried powdered adventitious roots of *Echinacea purpurea*. 60% ethanol was found to be suitable for

Table 1. Phenolics, flavonoids, and polysaccharides contents in the dried adventitious root extract of *Echinacea purpurea* as affected by the type and concentration of solvents used for heat reflux extraction

Solvent concentration (% v/v)	Total content (mg/g DW)		
	phenolics	flavonoids	polysaccharides
	units are expressed as gallic acid equivalents		
Water	25.5 f	12.5 e	52.9 bc
Methanol			
20	35.8 ed	19.3 d	43.4 b
40	36.4 ed	22.9 bc	40.6 g
60	47.1 b	32.6 a	53.5 a
80	51.6 a	32.8 a	53.8 a
100	14.1 g	3.3 f	50.3 cb
Ethanol			
20	33.7 e	23.2 c	47.3 ed
40	38.8 d	27.8 b	49.6 d
60	52.3 a	32.4 a	49.6 d
80	43.7 c	29.3 b	46.2 ef
100	11.6 g	1.5 g	44.1 f

Different letters within the columns indicate statistical differences according to Duncan's multiple range test at $P = 0.05$

Table 2. Contents of caffeic acid derivatives in the dried adventitious root extract of *Echinacea purpurea* as affected by the type and concentration of solvents used for heat reflux extraction

Solvent concentration (% v/v)	Caffeic acid derivatives (mg/g DW)		
	caftaric	chlorogenic	chichoric
Water	1.2 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
Methanol			
20	4.5 ± 0.1	1.5 ± 0.1	6.7 ± 0.1
40	4.9 ± 0.1	1.6 ± 0.1	12.9 ± 0.1
60	4.9 ± 0.1	5.0 ± 0.1	22.7 ± 0.2
80	4.9 ± 0.1	5.0 ± 0.1	23.9 ± 0.1
100	0.8 ± 0.1	0.2 ± 0.1	1.0 ± 0.1
Ethanol			
20	3.9 ± 0.1	1.9 ± 0.1	6.1 ± 0.1
40	4.7 ± 0.1	4.8 ± 0.1	21.3 ± 0.1
60	4.9 ± 0.1	5.4 ± 0.1	24.6 ± 0.1
80	1.2 ± 0.2	5.0 ± 0.1	16.2 ± 0.1
100	0.2 ± 0.3	0.2 ± 0.1	0.33 ± 0.1

Mean values of three replicates with relative standard deviations

the extraction of the active ingredients (Tables 1 and 2). Optimum yields of phenolics (52.3 mg/g DW), flavonoids (32.4 mg/g DW), polysaccharides (49.6 mg/g DW), caftaric acid (4.9 mg/g DW), chlorogenic acid (5.4 mg/g DW), and chichoric acid (24.6 mg/g DW) could be achieved with the use of 60% ethanol. When the ethanol concentration was higher than 60%, the contents of active ingredients decreased (Tables 1 and 2). The reason might reside in that proteins could be coagulated at higher concentrations of ethanol, causing a greater diffusion resistance. These results show that under the conditions tested, 60% ethanol was superior for dissolving the active ingredients of *E. purpurea*. Ethanol is not toxic, it is easy to be recycled, and is a preferred solvent for the extraction of the bioactive components from plant raw materials including ginseng (Kim *et al*. 2007).

The effect of extraction temperature

The extraction temperature is one of the important factors influencing the recovery the bioactive compounds during heat the reflux extraction (Wang & Weller 2006). The samples of *E. purpurea* adventitious root were extracted with 60% ethanol at 40, 60, and 80°C for two hours. The results showed that the temperature had a profound influence on the elution of the active ingredients from the samples (Tables 3 and 4). The treatment of the samples with 60% ethanol at 60°C yielded 53.4 mg/g DW phenolics, 33.1 mg/g DW flavonoids, 56.6 mg/g DW polysaccharides, 4.1 mg/g DW caftraric acid, 3.6 mg/g DW chlorogenic acid, and 28.8 mg/g DW chichoric acid. These increments in phenolics, flavonoids, polysaccharides, and caffeic acid derivatives with the increase of temperature might be the result of an increased diffusivity of the solvent into the cells and an enhanced desorption

Table 3. Phenolics, flavonoids, and polysaccharides contents in the dried adventitious root extract of *Echinacea purpurea* as affected by the solvent temperature during heat reflux extraction for 2 hours

Solvent temperature (°C)	Total content (mg/g DW)		
	phenolics	flavonoids	polysaccharides
40	52.9 ab	32.3 ab	50.5 b
60	53.4 a	33.1 ab	56.6 a
80	53.1 ab	34.4 a	52.4 b

Different letters within the column indicate statistical differences according to Duncan's multiple range test at $P = 0.05$

Table 4. Contents of caffeic acid derivatives in the dried adventitious root extract of *Echinacea purpurea* as affected by the solvent temperature during heat reflux extraction for 2 hours

Solvent concentration (%)	Caffeic acid derivatives (mg/g DW)		
	caftaric	chlorogenic	chichoric
40°C	3.4 ± 0.1	2.8 ± 0.4	21.6 ± 0.6
60°C	4.1 ± 0.1	3.6 ± 0.1	28.8 ± 0.5
80°C	4.4 ± 0.2	3.7 ± 0.1	0.4 ± 0.2

Mean values of three replicates with relative standard deviations

of the components forming the cells (Camel 2000; Kim *et al*. 2007).

CONCLUSIONS

In this study, the effects were studied of various solvents and of the temperature of the extraction solvent selected with the aim of finding the efficient extraction of active ingredients from the dried adventitious roots of *Echinacea purpurea*. Out of the different solvents [distilled water, methanol (20, 40, 60, 80, and 100%) and ethanol (20, 40, 60, 80 and 100%)] tested, 60% ethanol was found to be superior for the extraction of the bioactive ingredients from powdered roots. The solvent temperature also influenced the extraction process; of the different temperature regimes tested, 60°C operational temperature (solvent temperature) was optimal for the separation of active components.

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