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Full Length Research Paper

Study on the extraction process and tyrosinase inhibition property of cichoric acid in *Echinacea purpurea* L.

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Echinacea purpurea **has become a commonly used herbal medicine because it contains various biologically active compounds, such as cichoric acid. Extraction conditions of cichoric acid from the flowers of *E. purpurea* L. were investigated through an orthogonal design of L_9 (3^4) assay. For purification, the extract was adsorbed on an AB-8 macroporous resin and eluted with 50% ethanol, then extracted with ethyl acetate four times, resulting in a residue containing 60.23% cichoric acid. Tyrosinase inhibition property of cichoric acid extracts was tested compared with arbutin by Spectrophotometer method. The extraction was optimized by adding 40% ethanol at a ratio of 1:15 (g/mL) to extract three times at 70□C for 1 h. The cichoric acid extracts from *E. purpurea* was found to have significant tyrosinase inhibition activity in broad range of concentration (≥10 mg/mL).**

Key words: *Echinacea purpurea* L., cichoric acid; extraction, orthogonal design, antimicrobial activity.

INTRODUCTION

Echinacea purpurea L. or purple coneflower has become an important medicinal plant and has a long history of medicinal use for a wide variety of illnesses, such as snakebites, syphilis, septic wounds, blood poisoning, influenza, common cold and other upper respiratory tract infections (Hobbs, 1994; Melchart et al., 1998; Melchart et al., 2006; Barrett, 2003). It is widely grown in various parts of world for commercial purpose. Extracts from the plant have shown antioxidant, antibacterial, antiviral and antifungal properties, and are used as medicines, nutritional supplements and health foods in Europe and America (Speroni et al., 2002). As early as 1000 years ago, the American Indians first discovered this herb, which became an essential medicine for their tribes. Nowadays, *E. purpurea* is the most commonly consumed species in the United States and Australia. In Europe, it has been the top selling product for several years (Borchers et al., 2000; Wills and Stuart, 1999; Line et al., 2007; Percival, 2000).

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E. purpurea is commonly used as an herbal medicine because it contains diverse biologically active caffeic acid derivatives, particularly cichoric acid (Luo et al., 2003). Cichoric acid is found in the flowers, roots and rhizomes, and to a lesser extent, in the stems and leaves, of *E. purpurea*. Cichoric acid has immunostimulatory properties, and can stimulate phagocytosis *in vitro* and *in vivo*. It also has anti-hyaluronidase activity, antiviral activity, and has been shown to inhibit HIV-1 integrase and replication (Cech et al., 2006; Lin et al., 1999).

To date, studies of cichoric acid in *E. purpurea* have focused on the preparation of standard by HPLC (Bo et al., 2001), the determination and primary extraction (Wang et al., 2002; Zhong et al., 2010) of cichoric acid. Ultrasonic extraction (Cao et al., 2010) and ethanol reflux extraction (Zhong et al., 2010) are used to extract cichoric acid from *E. purpurea*. But the content of primary extract was low, the equipment of HPLC needs high quality condition and it is not easy to expand the scale of production. The activity of tyrosinase showed some relation to production of melanin (Kim and Uyama, 2005). Melanin plays the main role in skin colour and pigmentation, and up to 10% of skin cells in the innermost layer of the epidermis produce melanin (Momtaz et al.,

Table 1. Factors and levels.

Level	Factors			
	A (extraction temperature/℃)	B (solvent volume)	C (extraction time/h)	D (Reflux times/time)
1	60	1:10	1	1
2	70	1:15	1.5	2
3	80	1:20	2	3

2008). Use of tyrosinase inhibitors such as arbutin and kojic acid is becoming increasingly important in the cosmetic industry due to their anti-pigmenting effects. There was no study to investigate the tyrosinase inhibition property of the extract from *E. purpurea*. In the present study, we extracted the active component of *E. purpurea* flowers by vapor distillation and determined the optimal processing conditions. For purification, the extract was adsorbed on an AB-8 macroporous resin and eluted with ethanol, then extracted with ethyl acetate. The purity was determined by HPLC, and then we investigated the tyrosinase inhibition property of the extract. These have been the basis for developing and using cichoric acid.

EXPERIMENTAL

Materials

Dried flowers of *E. purpurea* were purchased from Wei Qing Yao Cai Co, Ltd.(Anhui, China) and the tyrosinase was by the center of Chinese standard material. The morphological characters of the flowers were consistent with the description given in The Pharmacopoeia of the People's Republic of China. All solutions were prepared with analytical grade chemicals. Acetonitrile and glacial acetic acid were HPLC grade from Fluka (Buchs, Switzerland). Standard cichoric acid (purity ≥ 98.9%) was purchased from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The AB-8 macroporous resin was from Hefei Rong Yu Biotechnology Co, Ltd. (Anhui, China). The HPLC system consisted of an Agilent 1100 (Agilent Technologies, USA), a vacuum degasser, a quaternary pump, a variable wavelength detector, an injector with a 20 µl loop, an autosampler, an automatic thermostatic column compartment and a computer with a Chemstation software program.

Extraction

Powdered flowers (20 g) were subjected to two extraction processes by reflux for 2 h with water, 20, 40, 60, 80 and 100% ethanol (200 ml each). The filtrates were combined and concentrated under reduced pressure, then transferred to a 100 ml volumetric flask to determine the amount of cichoric acid, using HPLC. The extraction parameters involved four factors: (A) extraction temperature (60, 70 and 80ºC); (B) solvent volume (×10, 15 and 20 ml of sample weight); (C) extraction time (1, 1.5 and 2 h); (D) reflux times (1, 2 and 3 times) (Table 1). Orthogonal assay designs L_9 (3^4) (Table 2) were applied to optimize the extraction conditions (Wanida et al., 2009).

Quantitative HPLC of cichoric acid

Exact amounts of cichoric acid (1.3, 2.6, 3.9 and 5.2 mg) were weighed and dissolved in 5 ml 40% ethanol to give serial concentrations. The concentrations of these compounds in the samples were calculated according to the regression parameters derived from the standard curves. The chromatographic conditions were; column: ZorbaxSB C18 (4.6 × 150 mm, 5 µm), oven temperature: 35℃: and the mobile phase consisted of acetonitrile in 1.0% glacial acetic acid (25:75 v/v). The UV detection wavelength was 327 nm, flow rate was 10 mL min^{-1}, and the injection volume was 20 µl. The equation $y = 0.0002x - 0.1034$ ($R^2 = 0.9927$) was derived from the internal standard (Wang et al., 2002).

Purification

Powdered flowers (200 g) were extracted in the best condition, and combined and dried under reduced pressure. The dry extract (50 g) was dissolved with 40% ethanol (total volume 1,000 ml) and passed through an AB-8 macroporous resin column (200 g) to be absorbed, purified and eluted with 50% ethanol. The eluant was concentrated to 1/10 volume, adjusted to pH 3.0, then extracted with ethyl acetate four times (Wu et al., 2004). The final solution was carefully evaporated just to dryness with a vacuum evaporator (water bath at 35℃). The purity, determined by HPLC, was 60.23%.

Preparation of sample solutions

Extracts (purity ≥ 60%) / standard substance(purity ≥ 98.9%) / arbutin compounds were dissolved in phosphate buffer solution(PBS, PH = 6.8) to final concentrations of 20mg/ml, 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml. Arbutin was used as a positive control.

Colorimetric tyrosinase inhibition assay

As shown in Table 3, four reaction mixtures were prepared. Tyrosinase solution (50 Units/L in PBS, pH 6.8), PBS and sample solutions were mixed well in microtubes (10 mL), incubated at 37℃ for 15 min. Then 1 mL of L-tyrosine (3 mmol/L in PBS, pH 6.8) was added to the mixture. Mix well and incubate microtubes at 37℃ for 60 min. The reaction mixtures were then determined at 475 nm (Zhang and Li, 2009), a blank control group with PBS was selected. Each test was done in triplicate. The percentage tyrosinase inhibition was calculated as the following equation: the % Inhibition = [1 - (Ac - Ad) / (Aa - Ab)] × 100% where Aa is absorbance at 475 nm with enzyme and without test sample, Ab is absorbance at 475 nm without test sample and enzyme, Ac is absorbance at 475 nm with enzyme and test sample, and Ad is absorbance at 475 nm with test sample and without enzyme.

Table 2. Results and analysis of L_9 (3^4) orthogonal design.

Group [a]	A: extraction temperature (℃)	B: solvent volume (mL)	C: extraction time (h)	D: Reflux times	Extraction rate (%)
1	1	1	1	1	0.4063
2	1	2	2	2	1.0157
3	1	3	3	3	0.7387
4	2	1	2	3	1.3354
5	2	2	3	1	1.1889
6	2	3	1	2	1.2918
7	3	1	3	2	1.0472
8	3	2	1	3	1.7157
9	3	3	2	1	0.6356
K1	2.1607	2.7889	3.4138	2.2308	
K2	3.8161	3.9203	2.9867	3.3547	
K3	3.3985	2.6661	2.9748	3.7898	
X1	0.7202	0.9296	1.1379	0.7436	
X2	1.272	1.3068	0.9956	1.1182	
X3	1.1328	0.8887	0.9916	1.2633	
R	0.4126	0.4181	0.1463	0.5197	

[a] Nine groups having different combinations of parameters during extraction.

Table 3. Compositions and volumes of reaction mixtures.

Composition	Volume/mL			
	a	b	c	d
Tyrosinase solution	1.0	0	1.0	0
sample solution	0	0	1.0	1.0
PBS	3.0	4.0	2.0	3.0
L-tyrosine	1.0	1.0	1.0	1.0
Total	5.0	5.0	5.0	5.0

Statistical analysis

All experiments were carried out in duplicate; the results are expressed as mean ± standard deviation. Data were analyzed with Windows SPSS. Differences between groups were analyzed by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Optimization of extraction conditions

Water, 20, 40, 60, 80 and 100% ethanol were used to examine the extraction efficacy of cichoric acid in Table 4. The highest yield of cichoric acid was obtained with 20, 40 and 60% ethanol. The extraction yield was 0.74, 0.85 and 0.69%, respectively. The water and 80% ethanol yielded cichoric acid extracts of 0.31 and 0.23%, respectively, but no cichoric acid could be detected in 100% ethanol. *E. purpurea* contains polyphenol oxidase (PPO), and ethanol can augment the reducing of activity of PPO (Nusslein et al., 2000). When extracting with water, cichoric acid was oxidized in a few minutes; the oxidation rate was reduced by increasing the ethanol fraction. On the other hand, the solubility of cichoric acid in ethanol solution decreased with increasing volume fraction. Through the experiment, we decide to make the 40% ethanol as the extraction solvent.

On the basis of our experimental results, an orthogonal experiment was used to optimize the extraction conditions. The orthogonal assay designs L_9 (3^4) is shown in Table 2; and the data analysis is shown in Table 5. The results of variance analysis show that the significance of factors for the highest yield of cichoric acid were in the order: A > D > B > C, and the optimal extraction conditions were $A_2B_2C_1D_3$: 40% ethanol, extraction temperature 70℃, three times for 1 h, adding 15:1 (v/v)

Table 5. Results of variance analysis.

Variations	Square sum of deviation	Degree of freedom	Mean square	*F value*	*P*
A: extraction temperature	0.4941	2	0.2471	0.1922	< 0.05
B: solvent volume	0.3186	2	0.1593	0.1239	
C: extraction time	0.0416	2	0.0208	0.0162	
D: reflux times	0.4314	2	0.2157	0.1677	

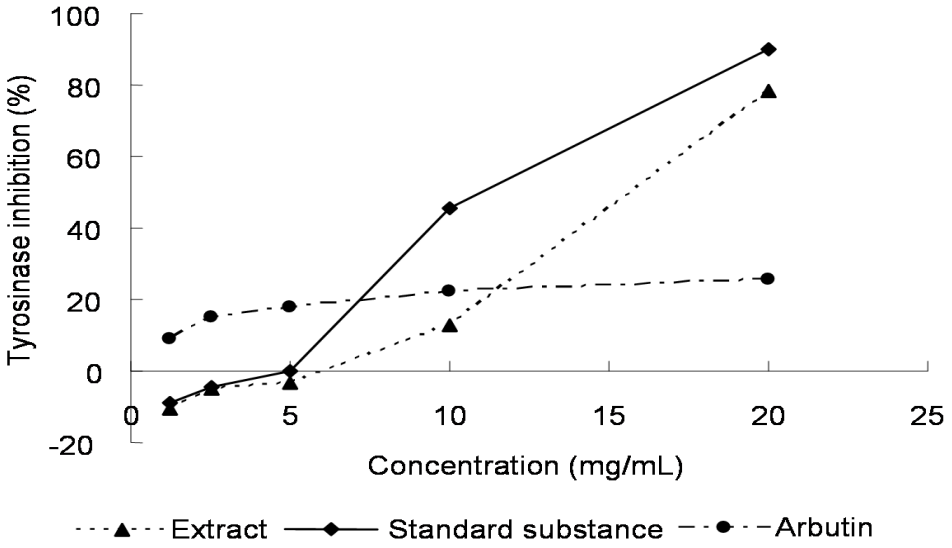

Figure 1. Tyrosinase inhibition of cichoric acid extracts.

with these best conditions, the purity, determined HPLC was 1.87%.

Tyrosinase inhibition activity

As shown in Figure 1, the extract and standard substance of cichoric acid has the similarity trend. They were both effective against tyrosinase in broad range of concentration (10-20 mg/mL), but low concentration (1.25-5 mg/mL) of them enhanced tyrosinase activity. Tyrosinase inhibition activity was strongest in cichoric acid (20 mg/mL standard substance: 89.79%; 20mg/mL extract: 78.25%), which was significantly higher than the positive control (20 mg/mL arbutin: 25.53%).

DISCUSSION

In this study, a simpler method has been introduced for extraction and determination of cichoric acid in detail. The purity of cichoric acid quantified by HPLC was 60.23%. F Zeng et al. (2004) used the method to separate and enrich the immunocompetence constituent cichoric acid from the extracts of *E. purpurea* with macroporous adsorption resin, by eluting with 7% (v/ v) $CH_3OH_2H_2O$. The cichoric acid content in product quantified by HPLC was nine times higher than that (4 %) of the extracts of *E. purpurea*. Wu et al. (2004) have reported that the best extraction conditions of cichoric acid from *E. purpurea* L. were 40% ethanol, extraction temperature 90ºC, three times for 2 h, adding 15:1 (v/v) ethanol , and the content was 1.12% at primary extracts. According to the study of Zhang et al. (2010), the optimum extracting conditions of cichoric acid from *E. purpurea* was showed as following: 50% ethanol, 55˚C, 3 h, adding 15:1 (v/v) ethanol, and the purity was about 1.3%. However, the purity in our study was about 1.9%. Therefore, our study can make the cichoric acid easy and swift to develop, and having certain practicability, moreover having reference for improving efficiency and reducing costs.

Compared with the positive control, cichoric acid extract showed stronger tyrosinase inhibitory than arbutin. For the group of standard substance, they showed stronger tyrosinase inhibitory activity than the extract group. According to our research, it would mean that cichoric acid studied had activity values that were significantly higher or comparable to the positive control. Cichoric acid extracts has not been explored previously for tyrosinase inhibitory activity. In conclusion, on the

basis of the experiment, we know some new information about tyrosinase inhibitory activity of cichoric acid in *E. purpurea*. However, a larger-scale study would be necessary to accurately determine the mechanism of action of cichoric acid.

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