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RAPID ISOCRATIC HPLC ANALYSIS OF CAFFEIC ACID DERIVATIVES FROM *Echinacea purpurea* CULTIVATED IN IRAN

M. Iranshahi[1] and Y. Amanzadeh[2]

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Cichoric acid and caftaric acid are the main phenolic compounds in Echinacea purpurea *tops. The level of these phenolic compounds in* E. purpurea *extracts is affected by different factors such as seasonal variations, drying methods, extraction methods, and growing location of the plant. HPLC analysis of caffeic acid derivatives in extracts of* Echinacea purpurea *(Cultivar) aerial parts, produced by boiling water extraction and ethanol-water extraction methods, showed various levels of the derivatives. Our findings revealed that the Iranian cultivated* E. purpurea *had a high level of cichoric acid (3.5-5.7 %). Caftaric acid was also the main phenolic compound in* E. purpurea *tops (3.1-4.5 %). After 2 h of boiling water extraction, the level of cichoric acid was 5.7 %, whereas the level of this acid in 60:40 ethanol-water extraction did not exceed 3.9 %.*

Key words: *Echinacea purpurea*, aerial parts, caftaric acid, cichoric acid, HPLC, extraction methods.

Within several herbs, *Echinacea* species are among the main investigation targets, because of their value as immunostimulants [1].

The therapeutic effect of *Echinacea* has been assigned to the presence of caffeic acid derivatives such as cichoric acid and lipophilic polyacetylene-derived compounds, such as alkylamides, constituting isobutylamides, and various other components found in the extracts. Cichoric acid has been shown to possess phagocytosis stimulatory activity *in vitro* and *in vivo* in addition to inhibiting hyaluronidase and protecting collagen type III free radical induced degradation [1]. Cichoric acid also has antiviral activity [2] and recently has been found to inhibit HIV-1 integrase and replication and also exhibits protective effects toward HIV-infected cells [3].

In the present study, we briefly reviewed different extraction procedures in previous works. We have also investigated the level of the caffeic acid derivatives in *Echinacea purpurea* cultivated in Iran by reverse phase HPLC.

Extraction of bioactive phenolic compounds from *Echinacea purpurea* was differentially affected by solvent composition. A previous study [4] showed that extraction of alkylamides and cichoric acid was differentially affected by ethanol composition, with 60 : 40 mixture of ethanol: water giving the maximum overall yield of both constituents. The Institute for Nutraceutical Advancement (a U.S. industry organization) method requires extraction of plant material with premixed 70 : 30 ethanol : water [5]. It was determined that addition of water to ground *E. purpurea* roots prior to adding ethanol resulted in the extract going brown within seconds. HPLC analysis showed loss of > 50 % of both cichoric acid and caftaric acid. Extraction with 70 % ethanol serves to denature this enzyme. Wills and Stuart [6] found that cichoric acid was lost if powdered *E. purpurea* got damp, but heating to denature the enzyme could prevent these losses. In another work [7] various extraction procedures of the *Echinacea purpurea* plant material were investigated in order to optimize the yield of phenolic compounds. The most useful method was extraction with methanol or ethanol, followed by extraction with dilute alcohols (60% v/v) after addition of water.

1) Department of Pharmacognosy and Biotechnology, Biotechnology and Pharmaceutical Research Centers, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran, fax (98511) 8823251, e-mail: iransham@sina.tums.ac.ir; 2) Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. Published in Khimiya Prirodnykh Soedinenii, No. 2, pp. 150-152, March-April, 2008. Original article submitted January 22, 2006.

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TABLE 1. HPLC Analysis of Phenolic Compounds in Aerial Part of *Echinacea purpurea* Cultivated in Iran by Different Methods of Extraction (t 60°C; Ethanol-Water 60:40; Material/Solvent - 1/7, w/v), %

Time, h	Acid, %[a]			Total polyphends, %[b]
	cichoric	caftaric	caffeic	
2.5	3.5[c]	4.5	0.8	8.8
4	3.9	4.0	1.0	8.9
5.5	3.9	4.1	1.1	9.1
8	3.9	3.6	1.1	8.6
24	3.8	3.1	1.0	7.9
1*	5.5	3.8	0.6	9.9
2*	5.7	4.4	0.8	10.9

[a]Individual phenolic compound/dried plant material ×100; [b]≈ cichoric acid (%) + caftaric acid (%) + caffeic acid (%); [c]mean, standard deviation was calculated on the basis of three times repetition of each experiment and was less than 0.01 for all assays.

*t 100° C, solvent – water, material/solvent – 1/10, w/v.

Almost the same results were obtained with boiling water. Therefore we chose these solvents for extraction procedures and investigated the levels of caffeic acid derivatives at different extraction times. After eight hours extraction with ethanol-water (60:40, 60°C), the maximum yield of total dried extract (18-20 % w/w, dried extract/dried plant) was obtained. A 5.5 h extraction time was found to be sufficient for the extraction of caffeic acid derivatives (Table 1).

This result supports the end point extraction measured for caffeic acid derivatives [3]. We have also found that 1 h boiling water extraction is sufficient for the extraction of phenolic compounds. Boiling water processing exhibited more total residue (40-45 % w/w, dried extract/dried plant) as compared with the ethanol-water extraction method. HPLC analysis of boiling water extract showed that bioactive components, specially cichoric acid, were extracted in greater quantities than in ethanol-water processing. It is obvious that more polysaccharides are extracted by boiling water compared to ethanol–water (60 : 40). However, the high residue of boiling water extraction may be attributed to more extraction of polysaccharides. The polysaccharides of *Echinacea* spp. activate phagocytosis and promote the production of interleukin 1 and interferon beta and the release of tumor necrosis factor. The above-mentioned factors have also been implicated in the activity of the aqueous extracts [8].

In spite of some advantages of the aqueous extraction method, including greater extraction of polysaccharides and caffeic acid derivatives than ethanol-water, the elevated temperatures used to inhibit polyphenol oxidase (PPO) activation is detrimental for alkylamides [4]. It should be pointed out that the pharmacological activities of *Echinacea* spp. have also been attributed to the essential oil, alkylamides, polyalkynes, and polyalkenes. These lipophilic component fractions in addition to caffeic acid derivatives appear to contribute to the immunostimulant activity of the alcoholic *Echinacea* extracts by stimulating phagocytosis of polymorphonuclear neutrophil granulocytes [8].

With respect to the standard level of cichoric acid *E. purpurea* aerial parts (1.2-3.1 %) [9], it was found that the Iranian cultivated *E. purpurea* has a high level of cichoric acid (3.5-5.7 %, Table 1) as well as other phenolics. In a study [9] the highest content of cichoric acid found was 4.67 %. In another investigation [5] cichoric acid was the main phenolic in *E. purpurea*, tops in mean percentages 2.02 % in summer and 0.52 % in autumn. Also, Bauer [10] showed that the content of cichoric acid varies more dramatically (0.0–0.4 %) than alkylamides, due likely to inconsistent inhibition of the enzymatic degradation of cichoric acid. It should be pointed out that only caffeic acid, caftaric acid, and cichoric acid were identified in HPLC profiles by comparison of retention times and UV profiles at 330 nm. According to the HPLC profile of the boiling water extraction method, cichoric acid was the predominant plant phenolic, constituting 52 and 55 % of the relative peak areas measured for caffeic acid derivatives. This result nearly supports the previous finding by Wills and Stuarts [6], who report that cichoric acid constituted 63 and 67 % of the relative peak area measured. In contrast, the caftaric acid content was 38 and 40 % of the peak area measured.This content was greater than previous reports (8–18 %) [3].

EXPERIMENTAL

^{1}H NMR spectra were measured in DMSO-d_6 with TMS as an internal standard using a Varian 400 Unity plus spectrometer. Melting points were taken on a Reichert-Jung apparatus and are uncorrected.

Plant Material, Drying Method and Extraction. Aerial parts of *Echinacea purpurea* (L.) Moench were collected at full bloom in the middle of August from Ardebil province north of Iran. Freshly gathered aerial parts were dried by air-drying at 50°C. Extraction procedures are shown in Table 1.

Extraction and Purification of Cichoric Acid as Standard. Cichoric acid was isolated and purified from the plant using the previously described protocol [11]. For this purpose, 1000 g *E. purpurea* aerial parts, roughly chopped, are boiled for 1 to 2 hour in 6 L water, with occasional stirring. The decoction, decanted and mixed with the liquid obtained by pressing the residue, is filtered and precipitated with a 20 % solution of neutral lead acetate. The gelatinous green lead salts are centrifuged, washed with water by centrifuging, suspended in 300 mL of water, and decomposed with hydrogen sulfide. When the precipitation of the lead is completed, the liquid is heated and filtered hot through a filter paper and the lead sulfide is washed with boiling water. The clear filtrate is concentrated under reduced pressure on steam-bath water. Two extractions with ether are sufficient to remove all of the cichoric acid, as shown by a chromatographic examination of the aqueous solution before and after extractions. After evaporation of the ether and drying under reduced pressure, an amorphous product is obtained. A 10 % aqueous solution gently heated and decolorized with charcoal deposited almost pure cichoric acid as needles.

The physical characteristics of the isolated cichoric acid were in accordance with those of the literature [11].

The ^{1}H NMR spectrum of the substance at 400 MHz also confirmed the structure.

HPLC Instrument and Chromatographic Conditions. HPLC was performed on a Waters system equipped with an RP-18 column (Nucleosil C_{18}, 5 mm, 250 × 4.6 mm) and a UV detector (Waters 486). The mobile phase was acetonitrile-water containing 0.1% phosphoric acid (70:30). Other chromatographic conditions: flow rate, 1.5 mL/min; wave length of detection, 330 nm; run time, 5 min; volume of injection, 50 mL.

Standard and Sample Solution. Cichoric acid was dissolved in EtOH–H_2O (7:3). The concentrations of the standard solution were 0.1 mg/mL, 0.03 mg/mL, 0.01 mg/mL, and 0.003 mg/mL, and then the calibration curve was established for cichoric acid. The extracts were also dissolved in EtOH–H_2O (7:3) and diluted by 1/625 mg/mL (extract/solvent) and injected into the HPLC instrument.

Calculation of Phenolic Compound Percentages. Three major peaks were recognized in the HPLC chromatograms of samples that were assigned to chicoric acid, caftaric acid, and caffeic acid with relative retention times 1, 0.46, and 0.7, respectively. According to the literature, caftaric acid (2-*O*-caffeoyltartaric acid) was eluted first, with caffeic acid following and cichoric acid (2,3-*O*-dicaffeoyltartaric acid) eluting last, thus reflecting the relative order of polarity of these three specific caffeic acid derivatives [2, 9, 12].

Quantification of cichoric acid, caftaric acid, and caffeic acid was carried out using the following equation and correction factors [5]:

% w/w Individual phenolic compounds = $[(a \times c \times 625)/M] \times 100$

a – concentration of phenolic compound (mg/mL) from linear regression analysis;

c – correction factors for caftaric acid (1.24, $MW_{cich.}/MW_{caft.}$) and caffeic acid (0.74, $MW_{cich.}/2 \times MW_{caff.}$, where 2 in this equation means that 2 molecules of caffeic acid have the same absorbance as 1 molecule of cichoric acid) against cichoric acid;

M – extract weight, mg.

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