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Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp.

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Abstract

The aim of this study was to set up and validate an RP-LC method with DAD-detection to quantify caffeic acid derivatives in various *Echinacea* spp. Samples were extracted with 80% methanol. The analyses were carried out on a Lichrospher RP-18 column (125 mm × 4 mm i.d., 5 μm), with a mobile phase gradient, which increases the acetonitrile level in a phosphoric acid solution (0.1%). The flow rate was 1.5 ml/min. Detection was set at 330 nm. This method allowed the identification and quantification of caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid in *Echinacea* roots and derivatives. The total phenolic content was 10.49 mg/g for *E. angustifolia*, 17.83 mg/g for *E. pallida* and 23.23 mg/g for *E. purpurea*. Among *Echinacea* commercial herbal medicines, a certain variability in the concentrations of phenolic compounds was observed.

The radical scavenging activity of *Echinacea* methanolic extracts was evaluated in vitro with a spectrophotometric method based on the reduction of an alcoholic 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical solution at 517 nm in the presence of a hydrogen donating antioxidant. As for pure compounds, echinacoside had the highest capacity to quench DPPH• radicals (EC_{50} = 6.6 μM), while caftaric acid had the lowest (EC_{50} = 20.5 μM). The average EC_{50} values for *E. purpurea*, *E. pallida* and *E. angustifolia* were 134, 167 and 231 μg/ml, respectively. The radical scavenging activity of *Echinacea* root extracts reflected their phenolic composition. The results indicate that *Echinacea* roots and derivatives are a good source of natural antioxidants and could be used to prevent free-radical-induced deleterious effects.

Keywords: *Echinacea*; Phenolic compounds; Caffeic acid derivatives; RP-LC; Radical scavenging; DPPH

1. Introduction

Echinacea spp. (family Asteraceae) herbal medicines and dietary supplements are traditionally used as immunostimulants in the treatment of inflammatory and viral diseases. The species employed are *E. angustifolia* DC (roots), *E. pallida* (Nutt.) Nutt. (roots), and *E. purpurea* (L.) Moench (roots and aerial parts).

The main active compounds of *Echinacea* spp. are alkamides and polyacetylenes [1], caffeic acid derivatives [2], polysaccharides [3] and glycoproteins [4]. With regard to caffeic acid derivatives, several compounds have been identified from the hydrophilic

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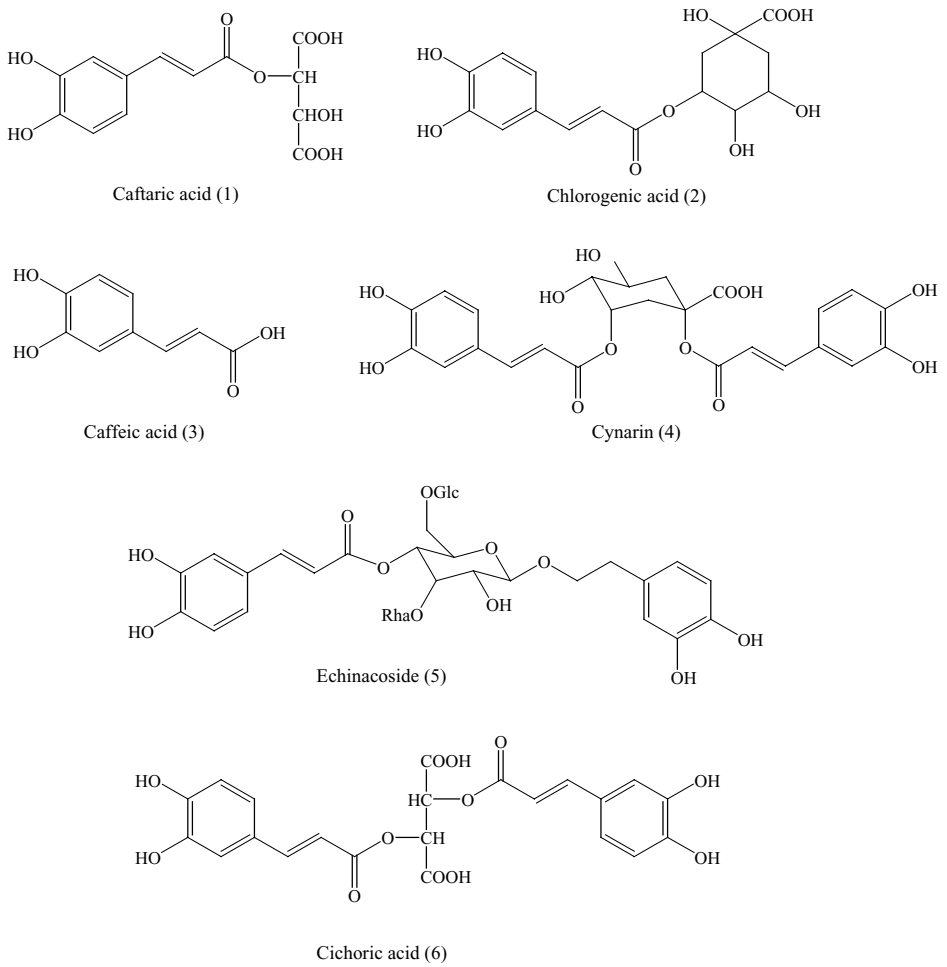

Fig. 1. Structure of caffeic acid derivatives of *Echinacea* spp.

fractions of *Echinacea* extracts, such as caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid (Fig. 1).

Cichoric acid is found to be the main phenolic compound in *E. purpurea*, but it does not occur in *E. pallida* and *E. angustifolia* in appreciable amounts [5]. Echinacoside is the main phenolic component in *E. angustifolia* and *E. pallida* roots [5]. Of the caffeic acid derivatives, only cichoric acid has shown immunostimulatory properties, promoting phagocyte activity in vitro and in vivo [6]. In addition, cichoric acid has antihyaluronidase activity [7], and has a protective effect on the free-radical-induced degradation of collagen [8]. Cichoric acid has also shown antiviral activity [2] and has recently been found to inhibit HIV-1 integrase and replication [9,10]. Echinacoside does not contribute to immunostimulant activity, but protects collagen against reactive oxygen species [8], and has antioxidant [11], antiinflammatory and cicatrizing activities [12].

Recently, there has been considerable interest in the nutraceutical industry and in preventive medicine in the quest for natural antioxidants from plant material. Various phytochemical components, such as flavonoids, phenylpropanoids and phenolic acids, are known to be responsible for the antioxidant capacity of fruits and vegetables. Consumers are now including phytonutrients in their diet, in the belief that antioxidant compounds may reduce the incidence of cancer, cardiovascular disease, arthritis,

and ageing in general, which are correlated with the damaging effects of uncontrolled free radical production.

Hydrophilic compounds of *Echinacea* spp. have been studied with several techniques, such as HPLC [5,13–18] and HPTLC [19,20]. MECK has been employed for the characterization of various hydrophilic components of *Echinacea* such as caffeic acid derivatives [21,22] and phenolic acids [23].

In view of the great commercial proliferation of *Echinacea* spp. herbal medicines in recent years and of the growing evidence of free-radical-induced deleterious effects, the present investigation was undertaken with the aim of analyzing the amounts of caffeic acid derivatives of *Echinacea* spp. Since the HPLC methods described in the literature for the quantification of phenolic compounds in *Echinacea* products were not completely validated, an RP-HPLC method with DAD-detection was set up and validated with the aim of quantifying the amounts of these components in various *Echinacea* spp. This technique was applied to evaluate the quality of *Echinacea* spp. roots and herbal medicines such as tablets, capsules and hydroalcoholic extracts.

With regard to antioxidant activity, the relationship between free radicals and the development of several degenerative diseases has led to considerable interest in assessing the antioxidant capacity of foods, medicinal plants and other nutritional antioxidant supplements. Antioxidant tests could be based on the evaluation of lipid peroxidation [24] or on the measurement of free radical scavenging ability [25]. Only occasionally have there been attempts to evaluate the antioxidant activity of *Echinacea* extracts [26,27]. In this study the radical scavenging activity of the plant extracts was evaluated against the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radicals. The contents of the phenolic compounds of *Echinacea* extracts were then correlated to their radical scavenging activity.

2. Experimental

2.1. Chemicals and reagents

Caftaric acid, chlorogenic acid, cynarin, echinacoside and cichoric acid were from ChromaDex (Laguna Hills, CA, USA). Caffeic acid, phosphoric acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) were from Sigma (Milan, Italy). Methanol, ethanol and acetonitrile HPLC grade were from J.T. Baker (Milan, Italy). Water was purified using a Milli-Q PLUS 185 system from Millipore (Milford, MA, USA).

2.2. Plant material

E. angustifolia DC (roots), *E. pallida* (Nutt.) Nutt. (roots) and *E. purpurea* (L.) Moench (roots) were harvested in spring 2003 from 3-year-old plants and were kindly donated by Dr. Nicola Aiello of the Forest and Range Management Research Institute (Villazzano, Trento, Italy). The roots were dried at 40 °C in a forced-air oven, since a higher temperature could cause loss of phenolics [28]. The dried roots were ground on a IKA M20 grinder (Staufen, Germany) before extraction.

Echinacea root herbal medicines were purchased from local markets in spring 2003. These samples include various formulations such as *E. angustifolia* hydroalcoholic extract (indicated as *E. angustifolia* herbal medicine), *E. pallida* capsules (indicated as *E. pallida* herbal medicines n.1 and n.2), and *E. purpurea* capsules (indicated as *E. purpurea* herbal medicine n.1) and tablets (indicated as *E. purpurea* herbal medicine n.2).

2.3. Sample preparation

A weighed amount (0.5 g) of ground dried roots was extracted with 10 ml of solvent at room temperature using a magnetic stirrer for 15 min. After centrifugation for 10 min, the supernatant solution was filtered under vacuum into a volumetric flask. The residue was re-extracted in the same way and the final volume of the solution was set at 25 ml. As for herbal medicines, a weighed amount (0.6–1.2 g of powdered tablets or the capsule contents) was extracted according to the procedure previously described. All the extracts were filtered through a 0.45 μm PTFE filter into a HPLC vial and capped.

The extraction procedure was repeated twice for each sample. *E. angustifolia* hydroalcoholic extract was filtered and directly injected in the HPLC system.

2.4. Choice of the best extraction solvent for LC analysis

To achieve the highest amount of antioxidants extracted from *Echinacea* roots, a range of different concentrations of methanol and ethanol aqueous solution (50, 60, 70, 80, 90 and 100%) was used following the above procedure. A higher percentage of water was not used to avoid hydrolysis of phenolic compounds [29].

2.5. Chromatographic apparatus

Chromatography was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system consisting of vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector (DAD).

2.6. LC determination of phenolic compounds

The analyses were carried out on a Lichrospher RP-18 column (125 mm × 4 mm i.d., 5 μm, Agilent Technologies). To protect the integrity of the analytical column, all analyses were performed with a coupled Lichrospher RP-18 guard column (4 mm × 4 mm, 5 μm, Agilent Technologies). The mobile phase was (A) aqueous phosphoric acid solution (0.1%) and (B) acetonitrile. The gradient elution was modified as follows: initial 10% B; linear gradient to 22% B in 13 min; recycle to initial conditions in 5 min and hold for 3 min. The total running time was 18 min. The post-running time was 3 min. The flow rate was 1.5 ml/min. The detector monitored the eluent at 330 nm. The column temperature was set at 26 °C. The sample injection volume was 5 μl. Three injections were performed for each sample.

2.7. Identification of constituents and peak purity

Peaks were identified on the basis of their retention time (t_R) values and UV spectra by comparison with those of the single caffeic acid derivative in the standard solution. Peak identity was also confirmed by spiking the extracts with pure standards.

Peak purity test was performed using a photo diode array detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic reference samples.

2.8. Calibration curves

The stock standard solution of each caffeic acid derivative was prepared as follows: about 2.0 mg of each compound was accurately weighed and placed into a 5 ml volumetric flask. Eighty percent methanol in water was added and the solution diluted to volume with the same solvent.

Calibration curves were established on five data points covering the concentration range of 12.89–386.60 μg/ml for caftaric acid, 15.64–391.00 μg/ml for chlorogenic acid, 12.85–385.40 μg/ml for caffeic acid, 15.99–399.78 μg/ml for cynarin, 19.64–392.80 μg/ml for echinacoside, 8.86–354.20 μg/ml for cichoric acid.

Five microliter aliquots of each standard solution were used for LC analysis. Triplicate injections were made for each standard solution. Each calibration curve was obtained by plotting the peak area of the phenolic compound at each level prepared versus the concentration of the sample.

2.9. Accuracy

The accuracy of the method was evaluated with the recovery test. This involved the addition of known quantities of caffeic acid derivative standards to known amounts of *Echinacea* roots. The fortified samples were then extracted and analyzed with the proposed HPLC method. The percentage recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100.

2.10. Precision of the chromatographic system

Intra- and inter-day precision was tested by performing multiple injections of a solution of all caffeic acid derivatives and then checking the percent relative standard deviation (%R.S.D.) of the retention times and peak areas. Ten injections were performed each day and this was repeated for 3 consecutive days.

2.11. Precision of the extraction procedure

The precision of the extraction procedure was validated using one *Echinacea* root sample (*E. purpurea*). Six samples, weighing about 0.5 g, were extracted as described above. An aliquot of each extract was then injected and quantified.

2.12. Limits of detection and quantification

Limits of detection (LOD) were calculated according to the expression $3.3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. Limits of quantification (LOQ) were established by using the expression $10\sigma/S$. LOD and LOQ were experimentally verified by injections of caffeic acid derivatives at the LOD and LOQ concentrations.

2.13. Stability of solutions

Stability was tested with standard solutions and sample solutions that were stored at 4 °C and at room temperature and analyzed every 12 h.

2.14. Determination of DPPH• radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) has been widely used to evaluate the free radical scavenging activity of natural antioxidants [25,30].

In this study, increasing aliquots of each extract were mixed with a methanolic solution of DPPH• (1 mM, 300 μl) in 4 ml cuvettes and brought to 3.0 ml with methanol. To eliminate the interference of the extract pigments with the DPPH• reaction, blanks of the extracts were performed using 300 μl of methanol instead of the DPPH• solution. After incubation in the dark at room temperature for 15 min, the spectrophotometric determination was assayed at 517 nm using a Lambda 5 UV-Vis spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). A DPPH• blank sample (containing 2.7 ml of methanol and 300 μl of DPPH• solution) was prepared and measured daily. The DPPH• solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at 4 °C between measurements. All experiments were carried out in duplicate and repeated at least twice.

The percent decrease in absorbance was recorded for each concentration, and percent quenching of DPPH• radical was calculated on the basis of the observed decrease in absorbance of the radical. % Inhibition/μl of extract change curves were used to find the concentration at which 50% radical scavenging occurred (EC_{50}). % Inhibition is calculated according to the formula:

$$\%\,\text{Inhibition} = \left(\frac{A_{\text{DPPH}} - A_{\text{Extr}}}{A_{\text{DPPH}}}\right) \times 100$$

where A_{DPPH} is the absorbance value of the DPPH• blank sample and A_{Extr} is the absorbance value of the test solution. A_{Extr} is evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank.

In order to evaluate the DPPH• radical scavenging activity of caffeic acid derivatives, this procedure was applied to solutions prepared by dissolving a weighed amount (1.4 mg) of pure standard in 10 ml of 80% methanol.

3. Results and discussion

3.1. Selection of the extraction solvent

To achieve the highest yield of extraction of phenolics from *Echinaea* roots, various concentrations of methanol and ethanol aqueous solution were used according to the extraction procedure described in the Section 2. Methanol/water extraction was more efficient than ethanol/water extraction for all three species of *Echinacea*. The effect of methanol concentration on the extraction efficiency of phenolics from *E. angustifolia*, *E. pallida* and *E. purpurea* roots is reported in Fig. 2a–c.

When the percentage of the organic solvent was 100%, the extraction efficiency was low. When the concentration of the organic solvent was from 90 to 70%, the extraction efficiency was high and did not vary significantly. When the concentration of the organic solvent was 60% or below, the extraction efficiency declined.

In particular, the most efficient solvent concentrations for the extraction of caffeic acid derivatives were: for *E. angustifolia*, 80% methanol followed by 90% methanol (Fig. 2a); for *E. pallida*, 80% methanol

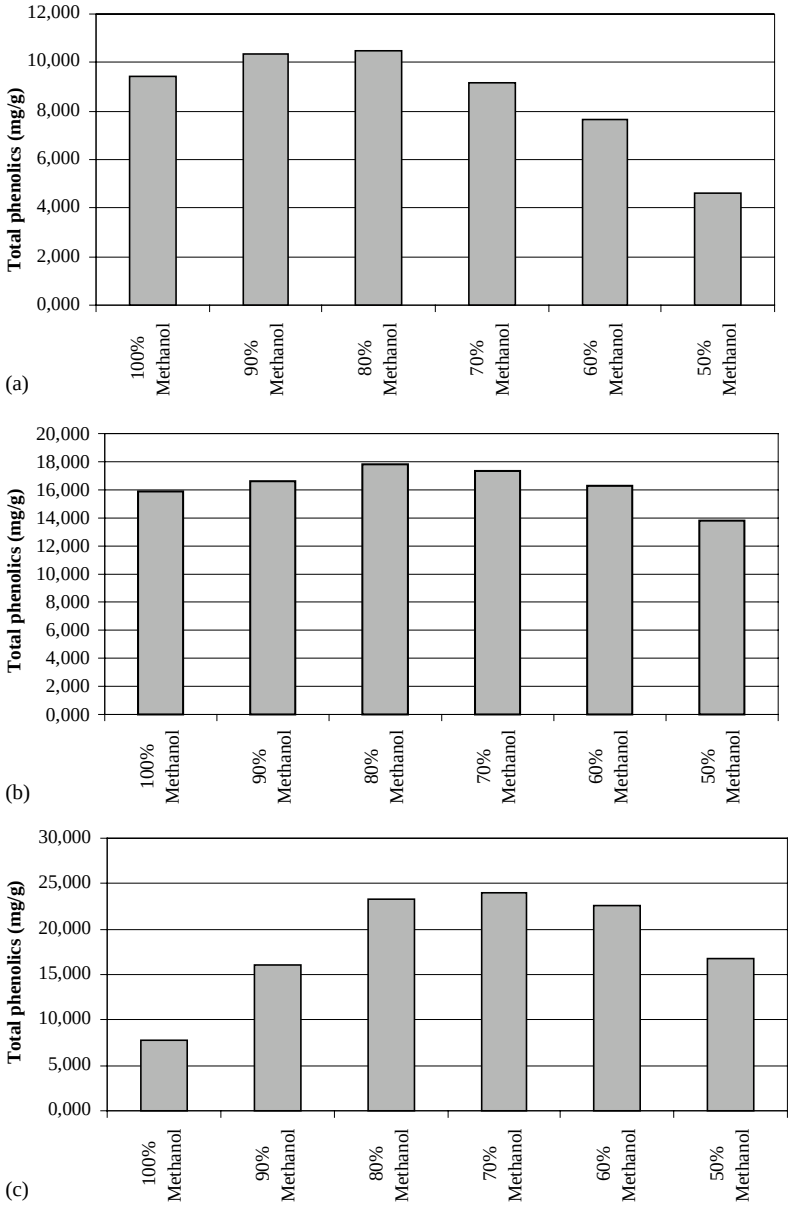

Fig. 2. Effect of different methanol concentrations on the extraction of total phenolics from (a) *E. angustifolia* roots, (b) *E. pallida* roots and (c) *E. purpurea* roots.

followed by 70% methanol (Fig. 2b); for *E. purpurea*, 70% methanol followed by 80% methanol (Fig. 2c). As reported in the literature, 70% methanol was the best solvent for the extraction of phenolics from *E. purpurea* roots [23].

Considering the results obtained for the three species of *Echinacea*, 80% methanol aqueous solution was finally chosen as the best extraction solvent for phenolic analysis in this study.

3.2. Optimization of the separation conditions

The chromatographic conditions were optimized with the aim of obtaining chromatograms with a good

resolution of adjacent peaks within a short analysis time.

Several mobile phases have been described in the literature for the analysis of caffeic acid derivatives in *Echinacea* roots using a reverse phase column [5,14–18,26,27]. In this study, two solvents were used as mobile phase: (A) water containing 0.1% phosphoric acid and (B) acetonitrile. Phosphoric acid was added because it reduced the peak tailing of caffeic acid derivatives [31]. Gradient elution was carried out so as to ensure that each run of analysis was completed within a short time. To optimize the mobile phase for a binary gradient profile, different compositions of acetonitrile in water containing 0.1% phosphoric acid were used. Under these gradient conditions (initial, 10% B; 0–13 min, 10–22% B; 13–18 min, 22–10% B) peaks were well separated in a short time.

Flow rates between 0.5 and 1.5 ml/min were studied. A flow rate of 1.5 ml/min gave an optimum signal to noise ratio with a reasonable separation time. The maximum absorption of caffeic acid derivatives was found to be at 330 nm, and this wavelength was chosen for the analysis.

Fig. 3 shows the chromatogram of mixed standards. A good resolution, with sharp symmetrical peaks, was achieved for all the phenolic compounds within 10 min when the solvent system and the chromatographic conditions reported in the Section 2 were employed. The retention times of caffeic acid derivatives are reported in Table 1.

Column performance results for all the phenolic compounds are presented in Table 1. As a measure of column performance, the number of theoretical plates (N) for caffeic acid derivatives was evaluated.

3.3. Suitability of the method

The chromatographic parameters (resolution, selectivity and tailing factor) were satisfactory for these compounds (Table 1). The calculated resolution values between each peak-pair were not less than 2.82 and the selectivity values were not less than 1.16.

3.4. Method validation

For the validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use and US Pharmacopeia 24 were followed [32,33].

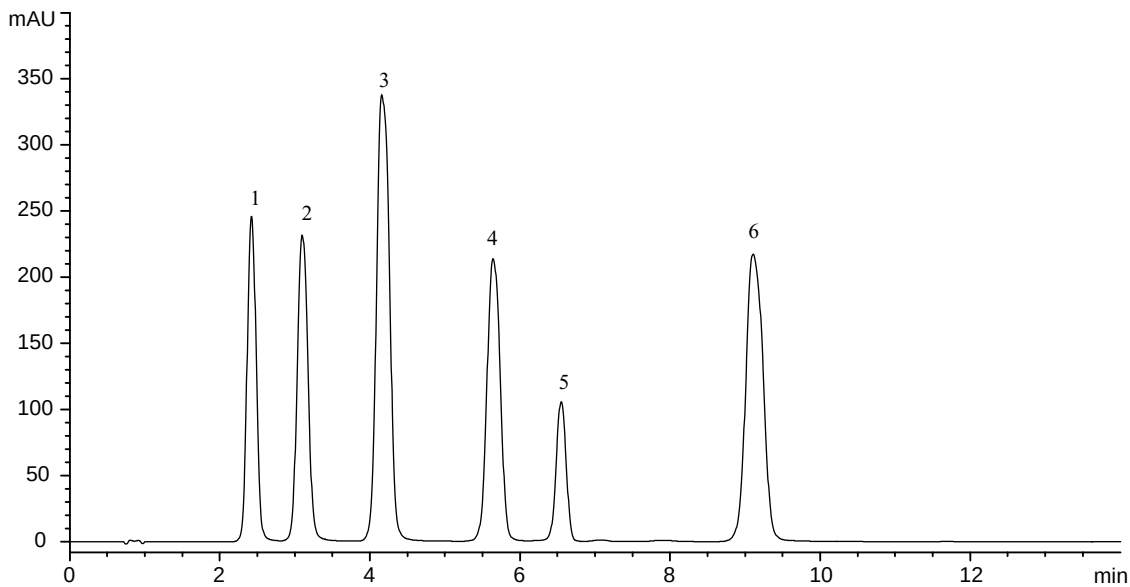

Fig. 3. HPLC separation of a standard mixture of caffeic acid derivatives. For peak identification, see Fig. 1.

Table 1
Column performance for the separation of caffeic acid derivatives

Compound	Retention time, t_R (min)	Number of theoretical plates, N	Resolution, R_s	Selectivity, α	Tailing factor, T
Caftaric acid	2.42	1,956	–	–	1.06
Chlorogenic acid	3.10	2,264	2.82	1.28	1.07
Caffeic acid	4.16	2,686	3.64	1.34	1.14
Cynarin	5.64	4,719	4.56	1.36	1.03
Echinacoside	6.55	11,043	3.13	1.16	0.97
Cichoric acid	9.11	7,349	7.57	1.39	1.11

Table 2
Statistical analysis for the calibration curves of caffeic acid derivatives[a]

Compound	Wavelength (nm)	Linearity range (μg/ml)	Slope, a	Intercept, b	r^2
Caftaric acid	330	12.89–386.60	8.800 (±0.015)	−2.066 (±2.628)	1.0000
Chlorogenic acid	330	15.64–391.00	9.869 (±0.022)	−21.514 (±3.855)	0.9999
Caffeic acid	330	12.85–385.40	17.488 (±0.036)	−21.515 (±6.189)	0.9999
Cynarin	330	15.99–399.78	10.617 (±0.030)	−18.523 (±5.346)	0.9999
Echinacoside	330	19.64–392.80	4.445 (±0.010)	−6.471 (±1.847)	0.9999
Cichoric acid	330	8.86–354.20	15.347 (±0.015)	−16.724 (±2.466)	1.0000

S.D. values are given in parenthesis.

[a] For each curve the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (μg/ml), a is the slope, b is the intercept and r^2 is the correlation coefficient.

3.4.1. Linearity

Linear regression analysis for each of the caffeic acid derivatives was performed by the external standard method. The validating parameters of each calibration curve (slope (a), intercept (b), correlation coefficient (r^2), standard deviation of the slope and standard deviation of the intercept) are described in Table 2. Excellent linearity was observed for all these compounds between peak areas and concentrations ($r^2 \geq 0.9999$) over the range tested.

3.4.2. Accuracy

The accuracy of the method was evaluated with the recovery test. Table 3 reports the recovery data which were obtained by comparing the results from samples and fortified samples. Considering the results of the recovery test, the method is deemed to be accurate.

3.4.3. Precision of the chromatographic system

Intra- and inter-day analyses of the same solution containing all the caffeic acid derivatives were used to validate the precision of the chromatographic system. Table 4 describes the %R.S.D. values of retention times and peak areas.

It was concluded that there was no significant difference for the analyses tested within day and between days.

Table 3
Results of the recovery test for caffeic acid derivatives from *E. angustifolia*, *E. pallida* and *E. purpurea* roots

Compound	Spiked amount (mg)	Recovery (%)	Mean ($n = 5$)	R.S.D. (%)
Caftaric acid	0.248	99.49–102.78	100.92	1.31
Chlorogenic acid	0.249	98.95–102.82	100.94	1.84
Caffeic acid	0.240	96.59–103.83	101.22	2.81
Cynarin	0.249	99.78–102.81	100.88	1.22
Echinacoside	0.235	98.20–102.35	99.85	1.71
Cichoric acid	0.241	98.71–102.25	100.27	1.45

R.S.D. (%) = (standard deviation/mean) × 100.

Table 4
Intra- and inter-day precision data for retention time (t_R) and area of caffeic acid derivatives

Compound	Intra-day precision				Inter-day precision			
	t_R (min) ($n = 10$, mean)	R.S.D. (%)	Area (mAU s) ($n = 10$, mean)	R.S.D. (%)	t_R (min) ($n = 30$, mean)	R.S.D. (%)	Area (mAU s) ($n = 30$, mean)	R.S.D. (%)
Caftaric acid	2.38	1.81	2112.80	1.08	2.40	2.30	2134.02	1.57
Chlorogenic acid	3.01	2.02	2386.45	1.17	3.03	2.42	2409.09	1.68
Caffeic acid	4.03	1.38	4121.70	0.84	4.07	1.85	4170.39	1.53
Cynarin	5.42	1.49	2660.74	0.99	5.51	2.07	2694.76	1.66
Echinacoside	6.32	1.61	1024.14	1.55	6.41	1.81	1034.84	1.67
Cichoric acid	8.29	0.62	3482.89	0.57	9.04	1.57	3524.32	1.30

3.4.4. Precision of the extraction procedure

The amounts of caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid in six sample solutions prepared from the same of *E. purpurea* root sample were 3.97 mg/g with an R.S.D. of 1.63%, 0.38 mg/g with an R.S.D. of 2.16%, 0.10 mg/g with an R.S.D. of 2.51%, 0.22 mg/g with an R.S.D. of 2.39%, 0.19 mg/g with an R.S.D. of 2.40%, 19.27 mg/g with an RDS of 1.35%, respectively. These results suggest that the method presented has good precision.

3.4.5. Limits of detection and quantification

LOD and LOQ were established by the procedures described in the Section 2. Table 5 shows the LOD and the LOQ values of caffeic acid derivatives.

3.4.6. Stability

The analytes in solution did not show any appreciable change in chromatographic profile for at least 72 h. No degradation products were detected.

Table 5
Limit of detection (LOD) and limit of quantification (LOQ)

Compound	LOD (μg/ml)	LOQ (μg/ml)
Caftaric acid	3.14	9.51
Chlorogenic acid	4.09	12.40
Caffeic acid	3.72	11.27
Cynarin	5.28	15.99
Echinacoside	3.78	11.46
Cichoric acid	1.69	5.13

3.5. Applications to Echinacea extracts

Fig. 4a–c show the chromatograms of the RP-HPLC analysis of caffeic acid derivatives in *E. angustifolia*, *E. pallida* and *E. purpurea* root extracts.

Echinacoside was found in the extracts of both *E. angustifolia* and *E. pallida* roots, whereas it was not detected in *E. purpurea*. *E. purpurea* root extracts contained a high amount of cichoric acid compared with the other two species. Cynarin was determined only in *E. angustifolia* roots. It has been suggested [5] that the presence/absence of cynarin could be used to distinguish *E. angustifolia* and *E. pallida*.

Table 6 reports the amounts of these compounds in *Echinacea* roots and herbal medicines. Data are expressed as mg/g of dry weight. The total phenolic content was 10.49 mg/g for *E. angustifolia*, 17.83 mg/g for *E. pallida* and 23.23 mg/g for *E. purpurea*. *E. angustifolia* had echinacoside as the major phenolic compound, followed by cynarin. In *E. pallida*, three phenolics were quantified: echinacoside, cichoric acid and caftaric acid. Cichoric acid and caftaric acid were the main phenolic compounds of *E. purpurea*. These data are within the range of those of previous reports [5,26]. Caffeic acid was not detected in these root samples because its level was below the LOD value. Chlorogenic acid was detected but not quantified since its concentration was below the LOQ value.

Table 6 also describes the results of the RP-HPLC analysis of *Echinacea* herbal medicines. The chromatogaphic profiles of these products were the same as those reported for *Echinacea* roots. However, there is a certain variability in the concentrations of phenolic compounds among the commercial samples of

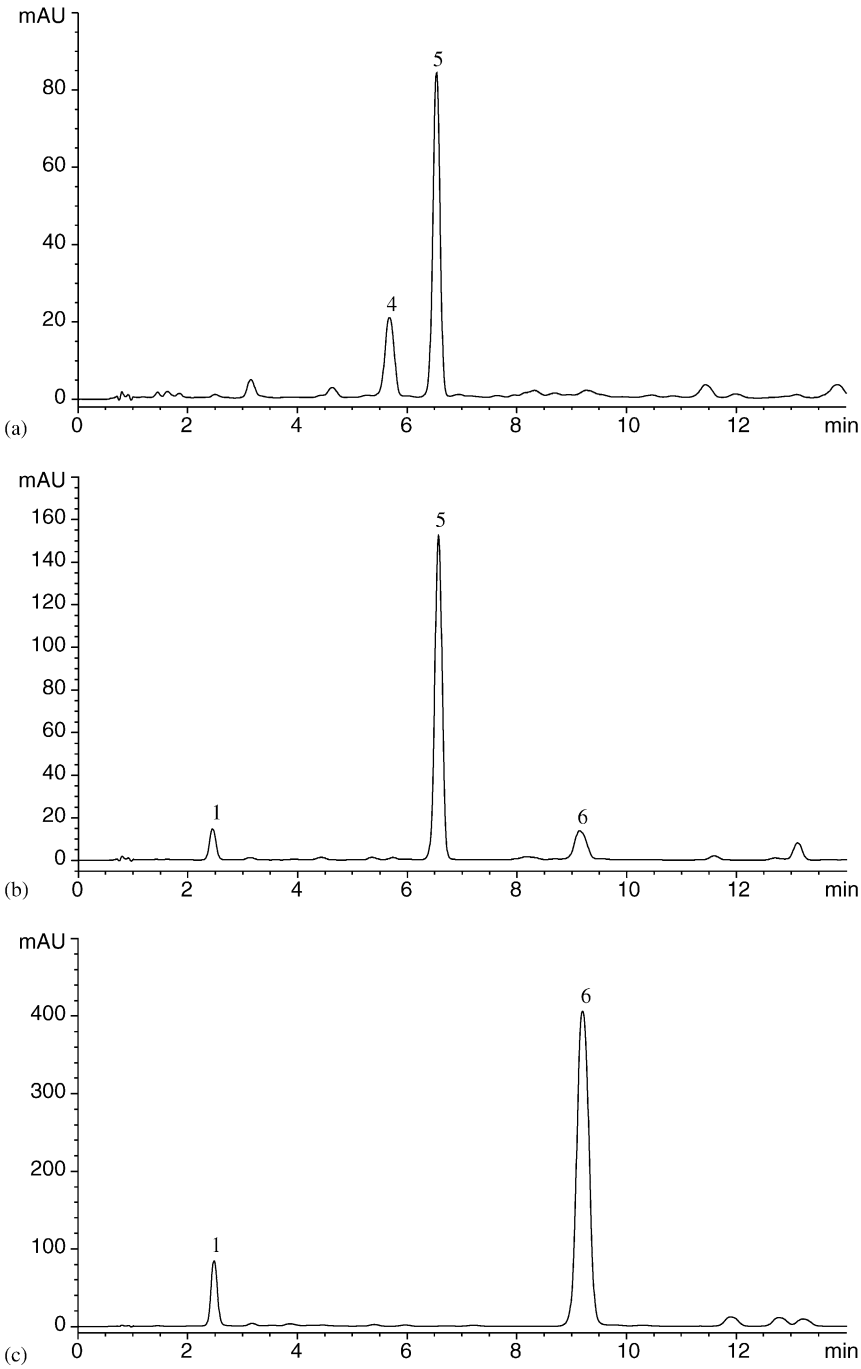

Fig. 4. HPLC analysis of caffeic acid derivatives of (a) *E. angustifolia*, (b) *E. pallida* and (c) *E. purpurea*. For peak identification, see Fig. 1.

Table 6
Content of caffeic acid derivatives in *Echinacea* spp. roots and herbal medicines by means of the RP-HPLC method

Sample	Content dry weight (mg/g)[a]						
	Caftaric acid	Chlorogenic acid	Caffeic acid	Cynarin	Echinacoside	Cichoric acid	Total phenolics
E. angustifolia	<LOQ	<LOQ	<LOD	1.39 ± 0.03	9.10 ± 0.20	<LOQ	10.49 ± 0.21
E. pallida	0.81 ± 0.02	<LOQ	<LOD	<LOD	16.18 ± 0.19	0.83 ± 0.03	17.83 ± 0.22
E. purpurea	3.97 ± 0.14	<LOQ	<LOD	<LOD	<LOQ	19.27 ± 0.26	23.23 ± 0.33
E. angustifolia herbal medicine[b]	<LOQ	<LOQ	<LOD	0.15[c]	1.08 ± 0.01	<LOQ	1.24 ± 0.01
E. pallida herbal medicine n.1	2.36 ± 0.04	<LOQ	<LOQ	<LOD	14.82 ± 0.19	1.24 ± 0.02	18.41 ± 0.26
E. pallida herbal medicine n.2	0.64 ± 0.01	<LOQ	<LOD	<LOD	4.17 ± 0.05	0.43 ± 0.01	5.23 ± 0.06
E. purpurea herbal medicine n.1	3.62 ± 0.08	<LOQ	<LOQ	<LOD	<LOQ	10.29 ± 0.22	13.92 ± 0.29
E. purpurea herbal medicine n.2	0.39 ± 0.02	<LOQ	<LOD	<LOD	<LOD	0.69 ± 0.03	1.08 ± 0.05

[a] Data are expressed as mean ± S.D. For each sample $n = 6$.
[b] Data are expressed as mg/ml.
[c] S.D. < 0.01.

Echinacea. In *E. angustifolia* herbal medicine the major phenolics were echinacoside and cynarin. In *E. pallida* herbal medicine n.1 there were high levels of echinacoside, caftaric acid and cichoric acid, while in *E. pallida* herbal medicine n.2 these amounts were smaller. In the case of the *E. purpurea* herbal medicines, the contents of cichoric acid and caftaric acid were high in *E. purpurea* herbal medicine n.1 and small in *E. purpurea* herbal medicine n.2.

According to the literature [14,18,22,23], a large range of concentrations of phenolic compounds has been observed for commercial products containing *Echinacea* species. Genetic variation and environmental factors, such as light, temperature, agronomic practices and so on, may have contributed to the differences in the level of caffeic acid derivatives between the various samples. In addition, drying temperature, extraction methods, formulations and storage conditions may have occasioned this variability.

3.6. DPPH• radical scavenging activity of pure compounds

The DPPH• radical scavenging activities of pure caffeic acid derivatives are reported in Table 7.

In the literature, the radical scavenging activity of phenolic compounds is described as being largely influenced by the number of hydroxyl groups on the aromatic ring [34,35]. The higher the number of hydroxyl groups, the greater the radical scavenging activity. The results of this study are in perfect agreement with these data. Echinacoside, cichoric acid and cynarin, with two adjacent hydroxyl groups on each of their phenolic rings, showed the highest radical scavenging activity. Chlorogenic acid, caffeic acid and caftaric acid with two adjacent hydroxyl groups on one ring showed lower antioxidant activity. In particular, the order of potency against DPPH• radicals was the following (Table 7):

echinacoside > cichoric acid > cynarin > chlorogenic acid > caffeic acid > caftaric acid

These results are in agreement with a previous study [8] which described the protective effects of these compounds against the degradation of collagen induced by oxygen radicals generated by the xanthine/xanthine oxidase/Fe^{2+}/EDTA system.

Table 7
DPPH• radical scavenging activity of caffeic acid derivatives

Compound	EC_{50} (μM)[a]
Caftaric acid	20.5 ± 2.4
Chlorogenic acid	18.9 ± 2.2
Caffeic acid	19.1 ± 1.9
Cynarin	11.0 ± 1.2
Echinacoside	6.6 ± 0.7
Cichoric acid	8.6 ± 0.9

[a] Radical scavenging activity expressed as mean ± S.D. ($n = 4$). EC_{50} is the value required to decrease the initial DPPH• concentration by 50%.

3.7. DPPH• radical scavenger activity of Echinacea roots and herbal medicines

Various phytochemical components, such as flavonoids, phenylpropanoids and phenolic acids, are known to be responsible for the antioxidant capacity of fruits and vegetables. Free radical scavenging is generally accepted to be the means by which antioxidant compounds inhibit lipid peroxidation.

The method employed [30] is based on the reduction of an alcoholic DPPH• solution at 517 nm in the presence of a hydrogen donating antioxidant (AH) due to the formation of the non-radical form (DPPH-H), according to the following reaction:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

The remaining DPPH•, measured after a certain time, correspond inversely to the radical scavenging activity of the sample. Through radical-radical interactions, the radical A• can contribute to the formation of stable molecules. This method is simple, rapid (15 min) and sensitive. No expensive reagents or sophisticated instrumentation are required.

The EC_{50} values of *Echinacea* roots and herbal medicines are described in Table 8.

The DPPH• scavenging activity was high in *E. purpurea* and decreased in *E. pallida* and *E. angustifolia*. These data are in agreement with those reported by Sloley et al. [27] who evaluated the antioxidant activity of *Echinacea* roots and leaves using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation model.

Table 8
DPPH• radical scavenging activity of *Echinacea* roots and herbal medicines

Sample	EC_{50} (μg/ml)[a]
E. angustifolia	231 ± 2.8
E. pallida	167 ± 1.6
E. purpurea	134 ± 0.7
E. angustifolia herbal medicine	7[b]
E. pallida herbal medicine n.1	191 ± 2.2
E. pallida herbal medicine n.2	104 ± 0.8
E. purpurea herbal medicine n.1	206 ± 2.3
E. purpurea herbal medicine n.2	2165 ± 20.9

[a] Radical scavenging activity expressed as mean±S.D. ($n = 4$).
[b] EC_{50} expressed as μl. S.D. < 0.1.

Data showed the existence of a correlation between the methanolic extract composition and the antioxidant activity. Of the three species tested, *E. purpurea* was the richest in total phenolics (Table 6) and its extracts proved the most effective at quenching free radicals (Table 8). *E. angustifolia* had the lowest content of total phenolics and its extracts displayed the lowest activity against DPPH• radicals. As for *E. pallida*, its values of both total phenolics and EC_{50} were intermediate.

The correlation coefficient between the DPPH• radical scavenging activity and total phenolic content was determined. The values of the DPPH• radical scavenging activity showed positive correlation with those of total phenolics: the correlation coefficient, r^2, was 0.991.

With regard to *Echinacea* herbal medicines, the EC_{50} values are reported in Table 8. Of the *E. pallida* formulations, herbal medicine n.2 had the lowest EC_{50} value, while herbal medicine n.1 had the highest. Of the *E. purpurea* formulations, herbal medicine n.1 displayed the highest radical scavenging activity, while *E. purpurea* herbal medicine n.2 had the lowest.

The EC_{50} values of *E. purpurea* medicines reflected their content of total phenolics. In the case of *E. pallida*, the radical scavenging activity did not follow the phenolic composition of the products. This could be due to the fact that herbal medicine n.2 contains a high amount of ascorbic acid, which could contribute to an overall synergic effect with phenolic compounds.

4. Conclusion

The RP-HPLC technique reported, using a diode array detector, is suitable for the analysis of caffeic acid derivatives. The method is simple, precise and economical in terms of time and solvent usage. A base line separation of all six compounds has been achieved. Through these phenolic markers, this method allows the unequivocal identification and standardization of the three most commonly used *Echinacea* species. The validation procedure confirms that this technique affords reliable analysis of these phenolic components and is appropriate for the quality control of complex matrices such as *Echinacea* roots and herbal medicines.

The radical scavenging activity of each of the three *Echinacea* root species appears to reflect their chemical profiles. Extracts of *E. purpurea* roots have greater free radical scavenging capacity than those of *E. pallida* or *E. angustifolia*. In view of the high radical scavenger activity of caffeic acid derivatives, *Echinacea* root extracts and herbal medicines could afford health benefits by preventing unwanted free-radical-induced oxidative reactions.

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