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Stabilization of Caffeic Acid Derivatives in *Echinacea purpurea* L. Glycerin Extract

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Recent work has shown that enzymatic degradation and oxidation of cichoric acid and other caffeic derivatives occurs in *Echinacea* preparations. However, very little is known as to the means of stabilizing these phytopreparations. To stabilize the glycerin extract of *Echinacea purpurea*, we have evaluated the effects of 3 natural antioxidants (citric acid, malic acid, and hibiscus extract) on the stability of the major caffeic acid derivatives (caftaric acid, caffeic acid, cichoric acid, and 2-*O*-feruloyl-tartaric acid). Chlorogenic acid, which normally occurs in an ethanol extract of *E. purpurea*, was not present in the glycerin extract. The caffeic acid derivatives, with the exception of 2-*O*-feruloyl-tartaric acid, were subject to degradation in the control sample. 2-*O*-Feruloyl-tartaric acid was stable during the whole testing period. All antioxidant treatments greatly improved the stability of caffeic acid derivatives. Stability was dependent upon the concentration of antioxidant added.

KEYWORDS: ***Echinacea purpurea*; stabilization; antioxidant; citric acid; hibiscus extract; malic acid**

INTRODUCTION

Echinacea purpurea is one of the best-selling phytomedicines in the United States (*1*). It is used as an immunostimulant and appears to act by stimulating T-cell production, phagocytosis, lymphocytic activity, and cellular respiration, and by inhibiting hyaluronidase activity (*2*). Caffeic acid derivatives (CADs), polysaccharides, and alkamides are thought to be among the compounds responsible for this complex mode of action (*3*). Among the CADs, cichoric acid has been shown to act as an immunostimulant by stimulation of phagocytosis (*4*). Cichoric and caftaric acids have also demonstrated an anti-hyaluronidase activity (*5*). Unfortunately not all CADs have been investigated.

Recent work has shown that cichoric acid and other caffeic acid derivatives are highly susceptible to enzymatic degradation and oxidation in aqueous and hydro alcoholic (55% EtOH) solutions (*6*−*8*). However, very little is known as to the means of stabilizing these phytopreparations. Nüsslein et al. (*6*) suggested that the CADs are degraded by polyphenol oxidases and that this oxidative process can be partially delayed by the addition of a combination of ascorbic acid and ethanol. Their experiment was done on pressed juice of above-ground parts of *Echinacea purpurea*.

In light of the seasonality of harvest and extraction of fresh *Echinacea purpurea*, and the prominence of this herb as a commercial phytomedicine, it is critical to stabilize the known bioactive components. We have, thus, investigated the effects of natural antioxidants on the stability of the caffeic acid derivatives (**Figure 1**) present in the glycerin extract of fresh *E. purpurea* roots.

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MATERIALS AND METHODS

Plant Material and Extraction. *Echinacea purpurea* L. was cultivated at the Tom's of Maine herb farm, Saxtons River, VT, from seeds collected from the previous year's crop. The original seeds had been purchased from Johnny's Selected Seeds (Albion, ME). The fresh roots were harvested in autumn 2000. One part of fresh-milled roots was mixed with 1.5 parts of aqueous glycerin 65% (w/v) and macerated for 1 week at room temperature in a closed, full, 50-gal drum. A sample of this production batch (#10007G2) was sent to Tom's of Maine (Kennebunk, ME) for analysis. The macerate was filtered through cheesecloth at room temperature and centrifuged. The same lot was used for all stability experiments to ensure the homogeneity of the sample at the beginning of the experiment.

Stability of Glycerin Extract. Amber bottles (75-mL) were filled with 50 mL of the echinacea extract, with headspace to allow oxidation to occur. Citric acid (Staley, Decatur, IL), malic acid (Penta, Livingston, NJ), or *Hibiscus sabdariffa* L. dried flower glycerin extract (plant/solvent (w/v), 1:10) (Tom's of Maine, Rockingham, VT) was added to the *Echinacea* glycerin extract after filtration. Citric acid and malic acid were both used in the concentrations (w/v) of 0.1, 0.3, and 0.5%. Hibiscus glycerin (v/v) extracts were used in concentrations of 5, 10, and 15%. The bottles were kept in an incubator at 25 °C. Each treatment was done in 3 replicates.

HPLC Analysis. For the HPLC analysis, one part of the sample was mixed with the same volume of HPLC-grade water, and the mixture was centrifuged. The supernatant was used for the HPLC analysis. The analyses were performed on an Agilent HPLC system (series 1100) with quaternary pump, UV/Vis detector (DAD), and automatic sample injector (Agilent Technologies, Burlington, MA).

Separation was achieved on an RP-18 column (LiChrospher 3 μm, 33 × 7 mm i.d., Alltech, Deerfield, IL). The following gradient, with eluent (A) H_2O + 0.05% TFA and eluent (B) MeCN + 0.05% TFA, was used: 0−1.5 min, 15% B, 2 mL/min; 1.6−4.9 min, 16% B, 2 mL/min; 5−7 min, 16−30% B, 3 mL/min; 7−10 min, 30−75% B, 3

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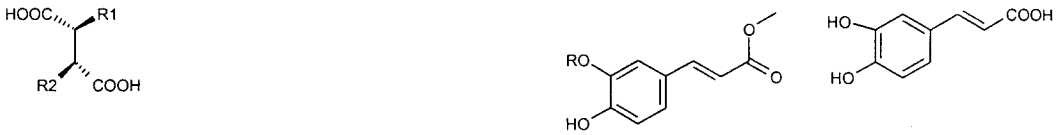

1 Caftaric acid R_1= caffeoyl, R_2=H

3 2-*O*-feruloyl-tartaric acid R_1=feruloyl,R_2=H

4 Cichoric acid R_1= R_2=caffeoyl

Caffeoyl R=H **2** Caffeic acid

Feruloyl R=Me

Figure 1. Caffeic acid derivatives in *Echinacea purpurea* root glycerite.

Table 1. Means and Relative Standard Deviations for Repeatability Tests

	mean (μg/mL)	RSD
caftaric acid	477.013	2.36
caffeic acid	50.319	1.92
cichoric acid	789.056	1.99

Table 2. HPLC–MS Analysis Data

retention time (min)	compound identity		fragment
2.90	caftaric acid	311 $[M - H]^-$	179 $[M - H -$ tartaric acid$]^-$
4.46	caffeic acid	163 $[M - OH]^+$	
5.58	2-*O*-feruloyl caftaric acid	325 $[M - H]^-$	193 $[M - H -$ tartaric acid$]^-$
7.85	cichoric acid	473 $[M - H]^-$	311 $[M - H -$ tartaric acid$]^-$

mL/min; 10 min, 75% B, 3 mL/min; 10.5−11 min, 100% B, 2 mL/min. The caffeic acid derivatives were quantified at 330 nm.

Linearity. The relation between peak area and concentration of standard had been found to be highly linear for caftaric acid, caffeic acid, and cichoric acid detected at 330 nm with r^2 values of 0.99998, 0.99991, and 1.00000, respectively.

Recoveries. The recovery experiment showed recovery rates of 99.5, 104.5, and 102.5% for caftaric acid, caffeic acid, and cichoric acid, respectively.

Repeatability. The repeatability was measured by performing 6 analyses of the same sample (**Table 1**).

HPLC−MS Analysis. The analyses were performed on an Agilent quaternary pump, UV/Vis detector (DAD), automatic sample injector, and an Agilent MSD Trap (Agilent 1100 series, Agilent Technologies, Burlington, MA).

Separation was achieved on the same HPLC column but the flow was adjusted to 0.9 mL/min to respond to the MS requirement. The appearance of the chromatogram stayed the same, but the elution was longer for every compound. Eluents A (H_2O + 0.05% TFA) and B (MeCN + 0.05% TFA) were used as follows: 0−1.5 min, 10% B; 3.1−4.25 min, 16% B; 7.5 min, 35% B; 8 min, 80% B; 12.5 min, 100% B. Flow rate was 0.9 mL/min. The caffeic acid derivatives were detected at 330 nm.

MS was conducted in the negative ion mode (except for caffeic acid (positive mode)) under the following conditions: scan range, 100−800 *m*/*z*; accumulation time, 16593 μs; skim 1, 25.0 V; capillary exit offset, 50.0 V; trap drive, 50.0 (**Table 2**).

Standards. Caftaric acid was purchased from Chromadex (Laguna Hills, CA). Cichoric acid was obtained from Addipharma (Hamburg, Germany). Caffeic acid and chlorogenic acid were obtained from Sigma Chemical Co. (St. Louis, MO). For each of those compounds a calibration curve was done, and interpolation gave the amount present. 2-*O*-Feruloyl-tartaric acid was quantified using the calibration curve obtained for caftaric acid.

Statistical Analysis. We subjected the treatment data to ANOVA, and then the statistical significance between the treatment and the control was assessed with Student's *t*-test (Microsoft Excel).

RESULTS AND DISCUSSION

The composition of a glycerite is slightly different from that of an alcoholic extract, and for this reason a new HPLC method has been developed (**Figure 2**). If we compare a 65% glycerin extract with one of 50% EtOH, the total content of caffeic acid derivatives (CAD) is similar, but the glycerin extract contains more cichoric and caftaric acids and less 2-*O*-feruloyl tartatric acid. The level of alkamides is higher in the ethanolic extract, but the content of polysaccharides is lower (*9*).

A preliminary experiment showed that the degradation of the caffeic acid derivatives was related to the level of air in the bottle. Practically, this indicates that oxidative degradation of the extract would be expected to accelerate in the opened bottle compared to that in the unopened bottle. To evaluate the influence of antioxidants added over a relatively short period of time, we chose in this experiment to observe the effects of several antioxidants on hydrophilic compounds in a half-full bottle. We acknowledge that the compounds we tested may have been more stable if we had used a full bottle because the enzymatic activity, as well as oxidation by the air, might have been reduced.

An LC−UV/MS−MS analysis was performed on the extract to ensure the identity of the compounds. Quantification of all of the compounds was performed by LC−UV. For caftaric acid, caffeic acid, and cichoric acid, we also did a spiking experiment to confirm the identities of the compounds. The identity of 2-*O*-feruloyltartaric acid was determined by the MS fragments. The ion mass appears at 325 $[M - H]^-$, and a fragment at 193 was representative of the feruloyl moiety $[\text{feruloyl} - H]^-$ after a loss of a tartaric acid. This substance has already been isolated from the leaves and aerial parts of *Echinacea purpurea* (*10*).

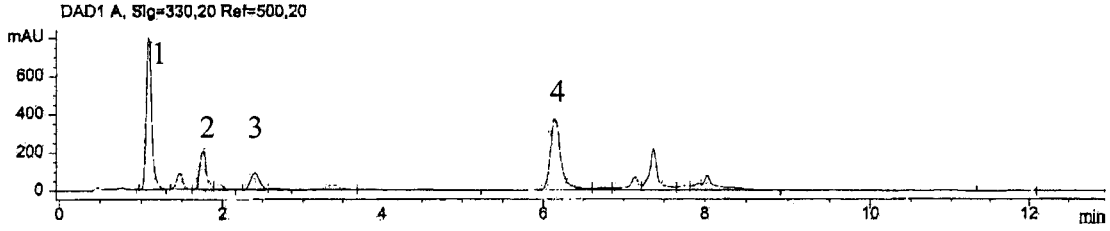

Figure 2. Chromatogram HPLC–UV of *Echinacea purpurea* root glycerite.

Table 3. Effect of Different Treatments on the Concentration of Caffeic Acid Derivatives in Glycerin Extract after 4 Months

treatment	caftaric acid mean (μg/mL) ± SD	caffeic acid mean (μg/mL) ± SD	2-*O*-feruloyl-tartaric acid mean (μg/mL) ± SD	cichoric acid mean (μg/mL) ± SD
control	357.9 ± 44.4	92.5 ± 18.4	246.3 ± 3.6	245.8 ± 35.6
citric acid 0.1%	607.1 ± 30.3	139.5 ± 7.9	263.6 ± 2.8	464.8 ± 23.4
citric acid 0.3%	998.7 ± 23.4	152.5 ± 6.5	284.1 ± 2.4	765.2 ± 12.7
citric acid 0.5%	1125.8 ± 23.1	138.8 ± 12.7	284.9 ± 2.4	854.1 ± 53.5
malic acid 0.1%	577.3 ± 34.4	99.8 ± 7.2	259.8 ± 1.6	383.1 ± 8.7
malic acid 0.3%	1017.7 ± 26.4	162.2 ± 30.3	289.8 ± 4.2	718.4 ± 60.9
malic acid 0.5%	1190.4 ± 36.8	180.0 ± 10.7	293.5 ± 1.3	864.4 ± 33.5
hibiscus 5%	579.2 ± 41.3	148.8 ± 9.7	252.7 ± 1.5	435.1 ± 8.7
hibiscus 10%	807.0 ± 37.2	170.4 ± 9.4	262.1 ± 0.2	573.4 ± 34.2
hibiscus 15%	1068.9 ± 39.7	152.7 ± 22.9	270.5 ± 4.6	701.8 ± 26.6

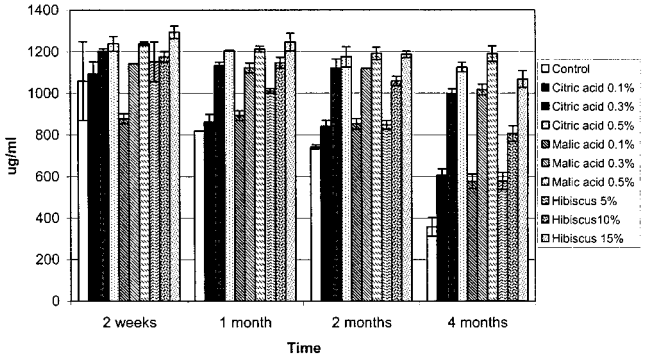

Figure 3. Concentration of caftaric acid in glycerin extract of *Echinacea purpurea* roots.

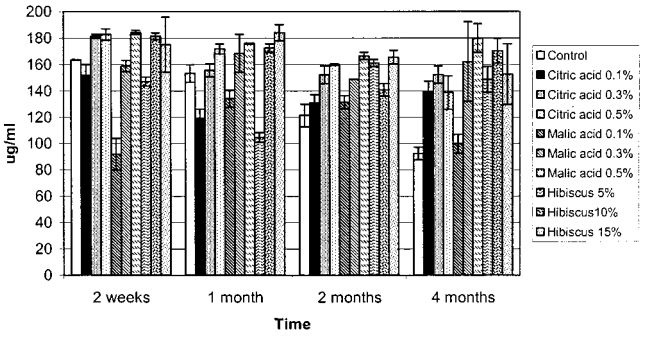

Figure 4. Concentration of caffeic acid in glycerin extract of *Echinacea purpurea* roots.

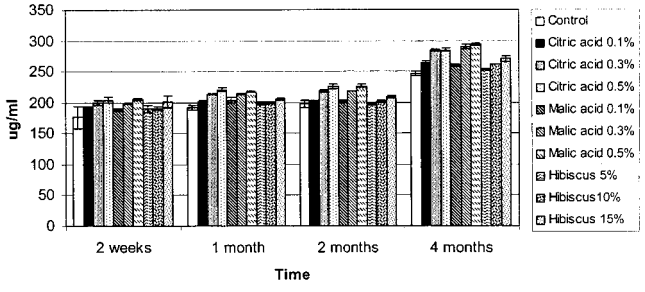

Figure 5. Concentration of 2-*O*-feruloyl-tartaric acid in glycerin extract of *Echinacea purpurea* roots.

As 2-*O*-feruloyl-tartaric acid was not commercially available, an evaluation had been done using caftaric acid as standard.

The effect of all treatments is obvious by the appearance of the sample. The treated samples are clearer and amber compared to the control which became progressively brown and opaque.

Figures 3, **4**, **5**, and **6** show the concentrations of caftaric acid (**1**), caffeic acid (**2**), 2-*O*-feruloyl-tartaric acid (**3**), and cichoric acid (**4**), respectively, in the root glycerin extract after

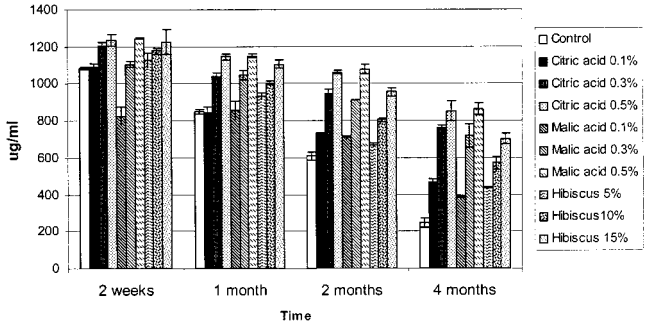

Figure 6. Concentration of cichoric acid in glycerin extract of *Echinacea purpurea* roots.

2 weeks, 1 month, 2 months, and 4 months of treatment. The rate of degradation in the control was significantly ($\alpha = 0.06$) higher than that in the treated samples after 4 months. The contents of **1**, **2**, and **4** dropped by 71.5, 49.9, and 80.4% respectively in the control after 4 months compared with the initial concentrations ($T = 0$). Compound **3** had increased by 21.2% during this time. At time 0, i.e., just after the filtration, the concentrations of **1**, **2**, **3**, and **4** were respectively 1257, 185, 203, and 1252 μg/mL.

The use of citric acid, malic acid, and hibiscus flower extract were all beneficial to the stabilization of **1**, **2**, and **4**, while **3** actually increased during the experiment (**Table 3**). A dose−response effect was observed, with the highest concentration of antioxidant resulting in the highest content of **1**, **2**, and **4**. However, the difference between citric acid and malic acid is not significant ($\alpha = 0.10$) for **1** and **4**.

After 4 months, the concentration of **4** in the control was 80.4% less than it was initially, whereas the treatments with citric acid (0.5%), malic acid (0.5%), and hibiscus extract (15%) resulted in losses of 31.8, 30.9, and 43.9%, respectively.

Addition of citric acid (0.5%), malic acid (0.5%), and hibiscus extract (15%) resulted in lower degradation rates of **1** (10.4, 5.29, 14.9%) and **2** (24.8, 12.1 and 17.2%) respectively, compared with their initial concentrations. Malic acid (0.5%) gave the best results for **1**, **2**, and **4**. The tartness of malic acid and citric acid, however, limits the concentrations that can be used in a commercial extract as it impacts the taste.

According to Nüsslein and collaborators (*6*), the degradation of cichoric acid is the result of oxygen from the air, and also enzymes such as polyphenol oxidase (PPO) and esterases. Cleaving the ester function by an esterase in **4** results in **1** and **2**. The quantities of **1** and **2** remain relatively constant during the experiment. This may be the result of the degradation of **1** and **2** coupled with an accumulation of **1** and **2** by either oxidation, or action of PPO or esterase of cichoric acid. The fact that **3** increases with time is possibly the result of the

esterase activity on 2-*O*-caffeoyl-3-*O*-feruloyl tartaric acid, a compound which is found in the extract but that has not been quantified.

Citric acid, a chelating agent, can complex metals such as copper and iron (*11*) in metal-catalyzed processes (*12*) including oxidation by enzymes like polyphenol oxidase. The carboxylic groups available in citric acid are responsible for this activity and can sequester the metals. We suppose that malic acid acts in a similar way as it also contains two carboxylic acids.

Previously, Duh and Yen (*13*) have reported the activities of phenolics as antioxidants in the calyx of *H. sabdariffa*. From our results, we believe that the presence of a high level (15−30%) of organic acids, including malic and citric acids (*14*), are also responsible for the results obtained with *Hibiscus sabdariffa*.

The results illustrate that the addition of malic acid, citric acid, or hibiscus extract can be used to stabilize the caffeic acid derivatives in the Echinacea glycerin extract. These compounds, and especially cichoric acid and caftaric acid, appear to be important for the immunostimulant activity (stimulation of phagocytosis and anti-hyaluronidase activity) of the plant, thus stabilization of the extract is very important to maintain a high quality and to ensure the efficacy over the course of treatment. These are not the only bioactive compounds, but the quantification of the other bioactive compounds (alkylamides and polysaccharides) is difficult as their standards are not commercially available. Certainly this line of study should be extended to the other caffeic acid derivatives, as well as the alkamides and the polysaccharides.

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