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CAFFEOYL CONJUGATES FROM *ECHINACEA* SPECIES: STRUCTURES AND BIOLOGICAL ACTIVITY

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Key Word Index—*Echinacea pallida*; Compositae; caffeic acid esters; quinic acid esters; tartaric acid esters; phenylpropanoid glycosides; high resolution ^{1}H and ^{13}C NMR mass spectrometry; antiviral activity; cell growth inhibition.

Abstract—Twelve caffeoyl conjugates were isolated from *Echinacea pallida* and their structures elucidated by NMR and mass spectrometry. The compounds are either caffeic glycosides or caffeic esters of quinic or tartaric acid. Three were previously unknown and five were isolated from this plant for the first time. The distribution of these compounds in the different organs of the plant is discussed. The antiviral and cell growth inhibitory effects of the major products are briefly reported.

INTRODUCTION

Echinacea angustifolia and *E. purpurea* (L.) Moensch are Compositae which have been used for a long time by native Indians of North America to cure wounds and snakebites. Nowadays. *Echinacea* species are used against several diseases [1–3], especially in Germany. Antiviral and immunostimulant effects have been shown [4–9] but the therapeutic activity of each species is not clearly defined as the drugs are generally prepared from mixtures of plants. It has been recently shown [10, 11] that, because of similarities in morphological characters, *E. angustifolia* and *E. pallida* Nutt. were often confused on the medicinal plant market. The chemical composition of these species has been extensively studied [12–24] and provides a good tool for distinguishing them. Results of the chemical analysis are also interesting in regard to the pharmacological activity and the toxicity of the extracts.

In a recent paper [25], caffeoyl derivatives were described as a 'new family of natural antiviral compounds'. Our present work deals with the isolation and structural elucidation of such derivatives from *E. pallida* Nutt. Commercial dried plant extracts were investigated and compared with extracts from fresh plants clearly identified as *E. pallida*.

RESULTS AND DISCUSSION

The methanolic extracts of *E. pallida* were partitioned with organic solvents. The ethyl acetate and *n*-butanol extracts contained the caffeoyl compounds and were fractionated on Sephadex LH 20. The different fractions, upon further preparative liquid chromatography or HPLC yielded pure compounds **1–12**.

Compounds **1–3** are quinyl esters of caffeic acid. Compound **1** was identified from its ^{1}H NMR spectrum as chlorogenic acid (5-*O*-caffeoylquinic acid), by comparison with an authentic sample. The structures of **2** and **3** were elucidated by ^{1}H NMR spectrometry. Compared with the spectrum of **1** (Table 1), the chemical shifts and peak intensities clearly show that **2** and **3** consist of quinic acid acylated with two caffeoyl moieties. The signals of H-3, H-4 and H-5 of the quinic acid moiety were assigned according to their multiplicity, their spin–spin coupling constants and by decoupling. In compound **2**, the H-5 chemical shift at 5.41 ppm is close to that of H-5 in chlorogenic acid whereas the signals of H-3 and H-4 are shifted downfield by 1.25 and 0.24 ppm respectively. On the basis of paramagnetic chemical shifts due to acylation, we conclude that in **2**, the hydroxyl group at C-3 is esterified by caffeic acid and that the compound **2** is therefore 3,5-*O*-dicaffeoylquinic acid. In the same manner, compound **3** was identified as 4,5-*O*-dicaffeoylquinic acid. In this case, a downfield shift by 1.4 ppm is observed for H-4 in the quinic moiety, whereas H-5 (5.66 ppm) and H-3 (4.39 ppm) are only slightly affected.

Compounds **4–8** are tartaric acid derivatives. Compounds **4** and **5** were previously described by Becker and Hsieh [22] and identified as 2,3-*O*-dicaffeoyltartaric acid (chicoric acid) and 2-*O*-caffeoyl-3-*O*-feruloyltartaric acid respectively, according to ^{1}H and ^{13}C NMR spectrometry. The ^{1}H NMR spectrum of **6** exhibits signals belonging to a caffeic moiety: two *trans* olefinic protons (7.59 and 6.28 ppm, $J = 15.9$ Hz) and three aromatic protons (ABX, 6.8–7.1 ppm), and two other doublets (1H each, $J = 2.4$ Hz) at 5.34 and 4.57 ppm. These results, as well as the ^{13}C NMR data (Table 2), are consistent with the structure of 2-*O*-caffeoyltartaric acid (caftaric acid).

Two new components **7** and **8** were isolated from the leaves. The identity of compound **7** was deduced from

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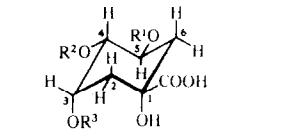
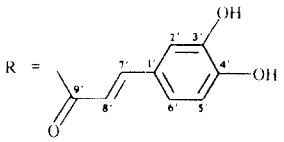
Table 1. ^{1}H NMR spectral data (400 MHz, CD_3OD) of quinic acid caffeic esters **1–3** [splitting patterns and J values (Hz) are given in parentheses]

H	1	2	3
2_{ax}	2.21 (*dd*, 13.6, 3.1)	2.26 (6.7)	1.9–2.2
2_{eq}	2.07 (*dd*, 13.6, 4.4)		
3	4.20 (*ddd*, 3.1, 4.4, 3.1)	5.5 (*dd*, 3.3–6.7)	4.39 (*ddd*, 3.0, 4.0, 3.1)
4	3.76 (*ddd*, 8.5, 3.1)	4.00 (*dd*, 7.5–3.3)	5.14 (*dd*, 3.0, 9.2)
5	5.37 (*ddd*, 9.3, 4.8, 8.5)	5.41 (*ddd*, 7.5, 3.6, 7.0)	5.66 (*m*, 13.0, 9.2, ~9)
6_{ax}	2.11 (*dd*, 13.6, 9.3)	2.19 (*dd*, 13.7, 7.0)	1.9–2.2 (*m*)
6_{eq}	2.26 (*dd*, 13.6, 4.8)	2.35 (*dd*, 13.7, 3.6)	
2′	7.08 (*d*, 2.0)	7.10/7.09 (*d*, 2.0)	7.03/7.05 (*d*, 2)
5′	6.81 (*d*, 8.2)	6.82/6.81 (*d*, 8.2)	6.77/6.78 (*d*, 8.2)
6′	6.98 (*dd*, 8.2, 2.0)	7.01/7.00 (*dd*, 2.0, 8.2)	6.93/6.95 (*dd*, 2.0, 8.2)
7′	7.59 (*d*, 15.9)	7.65/7.61 (*d*, 15.7)	7.62/7.54 (*d*, 15.9)
8′	6.29 (*d*, 15.9)	6.39/6.30 (*d*, 15.7)	6.31/6.22 (*d*, 15.9)

Table 2. ^{13}C NMR data (CD_3OD) of tartaric acid derivatives **4**, **6–8**

C	4	6	7	8
1	170.58	171.78	171.15	170.75
2	73.34	75.58	73.87	73.56
3	73.34	72.35	73.87	73.56
4	170.38	174.89	171.15	170.75
1′	128.59	128.49	128.57	
2′	116.08	116.31	116.05	
3′	147.65	147.28	147.75	
4′	150.76	150.36	150.79	
5′	117.40	117.58	117.45	
6′	124.29	124.38	124.27	
7′	149.42	149.40	149.29	
8′	114.17	114.68	114.85	
9′	169.59	169.24	168.71	
1″			127.67	127.64
2″			114.08	114.11
3″			148.87	148.93
4″			145.44	145.45
5″			133.61	133.61
6″			123.70	123.76
7″			149.20	149.47
8″			114.90	114.70
9″			168.75	168.62
1‴			128.55	128.66
2‴			118.11	118.12
3‴			147.57	147.56
4‴			146.78	146.78
5‴			117.03	117.05
6‴			122.30	122.31
7‴			39.74	39.75
8‴			49.12	49.20
9‴			178.55	178.55

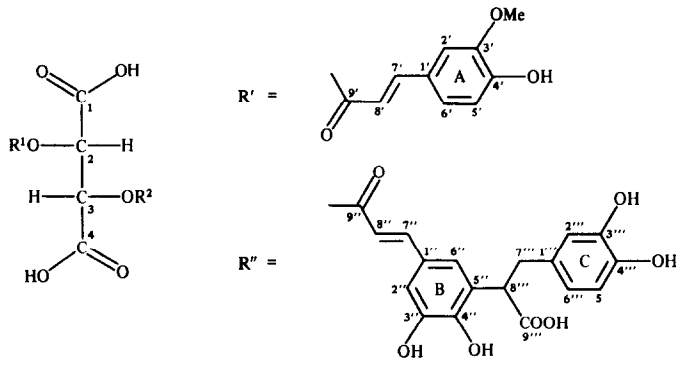




1 $R^1 = R$, $R^2 = R^3 = H$
2 $R^1 = R^3 = R$, $R^2 = H$
3 $R^1 = R^2 = R$, $R^3 = H$

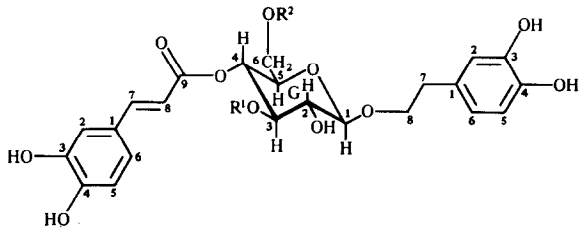
Fig. 1.

^{1}H NMR spectrometry. The assignment of the signals and the proton sequences were based upon a 2D homonuclear shift correlated (COSY) spectrum. These assignments were supported by negative ion mass spectrometry and ^{13}C NMR spectra. Compound **7** is an amorphous substance whose M_r is confirmed as 654 by secondary ion mass spectrometry (SIMS). The negative ion mass spectrum shows a weak $[M-H]^-$ signal at m/z 653 (rel. int.: 4). The ^{1}H NMR spectrum exhibits a signal at 5.70 ppm (2H) which could be attributed to H-2 and H-3 of 2,3,-*O*-diacyltartaric acid (cf. chicoric acid). The presence of a tartaric moiety was confirmed by the mass spectrum: peaks at m/z 149 and m/z 105 could be attributed respectively to $[\text{tartaric acid}-H]^-$ and $[\text{tartaric acid}-H-CO_2]^-$ fragments. The ^{1}H NMR spectrum suggested the presence of a caffeoyl group: two *trans* olefinic protons (7.5 and 6.3 ppm, $J = 16$ Hz) and three aromatic protons (ABX, 6.8–7.1 ppm). This was supported by a weak $[M-163]^-$ signal resulting from the loss of a caffeoyl moiety and by the more important fragments $[\text{caffeic acid}-H]^-$ at m/z 179 and $[\text{caffeic acid}-H-CO_2]^-$ at m/z 135. The ^{1}H NMR spectrum also demonstrated the presence of two other *trans* olefinic protons (7.5 and 6.3 ppm, $J = 16$ Hz) and two aromatic protons close to those of the caffeoyl moiety described above, with a *meta* coupling constant 1.6 Hz. These data were in good agreement with the presence of a 5-substituted *trans*-caffeoyl group. Three upfield aromatic protons (ABX, 6.4–6.6 ppm) could also be observed as well as three aliphatic protons (AMX, 2.79 ppm, *dd*, 7.2 and 14 Hz; 3.07 ppm, *dd*, 8 and 14 Hz; 4.11 ppm, *dd*, 8 and 7.2 Hz). The signal at 2.79 and 3.07 ppm suggested the presence of two geminal benzylic protons whereas the signal at 4.11 ppm could be attributed to a proton from a benzylic carbon atom bearing an electron withdrawing substituent such as –COOH. Therefore, we propose for **7** the structure shown in Fig. 2. The presence of the 5-[α-carboxyl-β-(3,4-dihydroxyphenyl)ethyl]caffeoyl group (R″) was supported in mass spectrometry by the [R″OH



4 $R^1 = R^2 = R$ (caffeoyl)
5 $R^1 = R, R^2 = R'$
6 $R^1 = R, R^2 = H$
7 $R^1 = R, R^2 = R''$
8 $R^1 = R^2 = R''$

Fig. 2.



9 $R^1 = R^2 = H$
10 R^1 = Rha, $R^2 = H$
11 R^1 = Rha, R^2 = Glc (G')
12 R^1 = Rha, R^2 = 6-caffeoyl-Glc

Fig. 3.

$-H]^-$ signal at *m/z* 359. The $[R''OH-H-CO_2]^-$ ion at *m/z* 315 and the $[M-R'']^-$ fragment at *m/z* 311. The ^{13}CNMR spectrum exhibits four distinct C=O signals between 168 and 179 ppm respectively attributable to C-1/C-4 from the tartaric moiety (171.15 ppm), C-9′/C-9″ (168.71/168.75 ppm) from the two ester linkages (cf. ^{13}C NMR data from chicoric acid: Table 2). The peak at 178.55 ppm confirms the presence of a fifth carbonyl group and was assigned to C-9‴ from the R″ carboxyl group. The allocation of the other carbon signals was made from a ^1H–^{13}C heteronuclear correlation. These data are given in Table 2 and can be compared with the ^{13}CNMR data of chicoric acid. The upfield shift by 16.2 ppm at C-5″ is in good agreement with an alkylation at C-5 in a caffeoyl moiety. However, the position of the carboxyl group (C-9‴) could not be unambiguously deduced from the NMR data and it was not clear from the results whether the –COOH was attached at C-8‴ or at C-7‴. The structure where –COOH is linked to C-8‴ was chosen on the basis of mass spectrometry data. A weak signal at *m/z* 237 could be attributed to [R″OH-3,4-dihydroxybenzyl]$^-$ and a most important ion at *m/z* 193, corresponding to the loss of CO_2 from the former, was observed. Finally NOE spectra were recorded. Irradiation at 4.11 ppm (H-8‴) clearly enhances the intensity of H-7‴, H-2‴ and H-6‴ signals; irradiation at H-7‴ enhances the intensity of H-8‴ and slightly affects the aromatic protons at the B ring. According to stereochemical considerations, these results are consistent with the structure of 2-*O*-caffeoyl-3-*O*-5[-α-carboxy-β-(3,4-dihydroxyphenyl) ethyl] caffeoyltartaric acid for **7**. It should be noted that the ^1H NMR spectrum of **7** varies with the experimental conditions, especially with concentration and with the presence of acid (trifluoracetic acid, TFA) in solution. The H-2″ and H-6″ protons are particularly affected and a downfield shift of 0.5 ppm was observed for one of these protons by adding two drops of TFA to a rather dilute solution of the compound in DMSO. These observations can probably be attributed to change in the molecular conformation according to weak intramolecular or intermolecular interactions.

The stereochemistry of tartaric acid was elucidated for chicoric acid only. it was found that **4** is the laevorotatory enantiomer, resulting from acylation of L(+)-tartaric acid as shown in ref. [26].

The structure of compound **8** was deduced from that of **7** by comparison of NMR spectra. The ^1H NMR spectrum of **8** exhibits a singlet at 5.70 ppm which suggested a 2,3-*O*-diacyltartaric acid. The other signals are similar to those previously described for R″. These data. together with the integration curve of the different proton signals, are consistent with the acylation by R″ of both –OH in tartaric acid. Therefore, **8** is 2,3-*O*-di 5-[α-carboxy-β-(3,4-dihydroxyphenyl)ethyl] caffeoyltartaric acid; this structure was supported by ^{13}CNMR data as shown in Table 2.

Compounds **9**–**12** are phenylpropanoid glycosides. Compounds **9**–**11** were extracted from *E. pallida* flowers. Compound **9** was isolated as an amorphous white powder. The ^1H NMR spectrum exhibits the signals of a caffeoyl moiety: two *trans* olefinic protons (7.49 and 6.24 ppm, $J = 15.9$ Hz) and three aromatic protons (ABX, 6.77–7.05 ppm). It also shows three upfield aromatic protons (ABX, 6.49–6.64 ppm) and four protons which absorb as an AMXY system at 2.69 (XY), 3.62/3.68 ppm (AM) respectively. These data are consistent with the presence of a β-(3,4-dihydroxyphenyl)ethoxy group and in good agreement with the results previously described

Table 3. ^{13}C NMR (DMSO) of phenylpropanoid glycosides **9**, **11** and **12**

C	9	11	12
Cafeic acid moiety			
1	126.15	125.53	125.71/125.76
2	115.43	114.79	115.21/115.66
3	146.16	145.61	145.68/145.54
4	149.00	148.59	148.66/148.52
5	116.39	115.80	115.94/115.99
6	121.99	121.88	121.69/121.65
7	146.00	145.90	146.05/145.68
8	114.61	113.50	113.58/114.01
9	166.54	165.80	166.76/166.23
Aglycone			
1	129.81	129.26	129.49
2	116.90	116.41	116.58
3	145.55	144.96	145.05
4	144.11	143.55	143.63
5	116.06	115.05	114.90
6	120.16	119.62	119.81
7	35.88	35.10	35.20
8	70.82	70.44	70.69
Glucose G			
1	103.40	102.22	102.47
2	74.19	74.75	74.62
3	75.22	78.95	79.15
4	71.89	69.13	69.30
5	74.67	73.18	73.23
6	61.42	68.05	68.29
Glucose G′			
1		103.42	103.42
2		73.79	73.55
3		76.88	76.37
4		69.96	69.95
5		76.50	73.99
6		61.05	63.60
Rhamnose			
1		101.27	101.44
2		70.44	70.67
3		70.44	70.54
4		71.71	71.83
5		68.81	69.01
6		18.18	18.33

for such compounds [27–28]. The assignment of the sugar protons was made from a 2D homonuclear (COSY) spectrum and demonstrates the presence of a β-D-glucose moiety acylated at C-4 as shown by the H-4 chemical shift (4.71 ppm). The ^{13}C NMR data confirm the structure of **9** as β-(3,4-dihydroxyphenyl)-ethyl-*O*-4-*O*-caffeoyl-β-D-glucopyranoside (desrhamnosylverbascoside). The ^{13}C shifts were assigned from a ^{1}H–^{13}C heteronuclear correlation and are comparable to the results previously described [29] except for the sequence between 113 and 117 ppm where several inversions are observed (Table 3).

Compounds **10** and **11** were identified as β-(3,4-dihydroxyphenyl)ethyl-*O*-α-L-rhamnopyranosyl(1→3)-4-*O*-caffeoyl-β-D-glucopyranoside(verbascoside) and β-(3,4-dihydroxyphenyl-ethyl-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucopyranoside (1→6)-4-*O*-caffeoyl-β-D-glucopyranoside(echinacoside) respectively, from their published NMR data [20, 27].

Table 4. ^{1}H NMR data (DMSO-d_6, 2 drops TFA; 400 MHz) of echinacoside **11** (20) and 6-*O*-caffeoylechinacoside **12** [splitting patterns and *J* values (Hz) are given in parentheses]

H	11	12
Caffeoyl moiety		
2	7.05 (*d*, 2.0)	7.06/6.90 (*d*, 2.0)
5	6.81 (*d*, 8.0)	6.77/6.73 (*d*, 8.0)
6	6.99 (*dd*, 2.0, 8.0)	6.99/6.89 (*dd*, 2.0, 8.0)
7	7.50 (*d*, 15.8)	7.48/7.44 (*d*, 15.8)
8	6.23 (*d*, 15.8)	6.26/6.18 (*d*, 15.8)
Aglycone		
2	6.67 (*d*, 2.0)	6.63 (*d*, 2.0)
5	6.66 (*d*, 8.0)	6.62 (*d*, 8.0)
6	6.54 (*dd*,2.0, 8.0)	6.48 (*dd*, 2.0, 8.0)
7	2.73 (*m*, 7.5)	2.67 (*dd*, 7.7, 7.3)
8	3.67; 3.92 (*m*)	3.59 (*m*, 8.8, 7.3); 3.86 (*m*, 8.8, 7.7)
Glucose G		
1	4.39 (*d*, 8)	4.34 (*d*, 7.7)
2	3.26 (*dd*, 8, 8)	3.21 (*dd*, 7.7, 9.0)
3	3.75 (*dd*, 8, 9.5)	3.73 (*dd*, 9.0, 9.5)
4	4.76 (*dd*, 9.5, 9.5)	4.71 (*dd*, 9.5, 9.5)
5	3.75 *m*)	3.75 (*m*)
6	3.54; 3.70	3.48 (*dd*, 6.8, 11.7); 3.70 (*m*)
Glucose G′		
1	4.22 (*d*, 8)	4.24 (*d*, 7.7)
2	2.99 (*dd*, 8, 8)	2.99 (*dd*, 7.7, 8.5)
3		3.13 (*dd*)
4	3.06–3.20	~3.10 (*dd*)
5		3.33 (*m*)
6	3.44; 3.67 (*m*, 12)	4.09 (*dd*, 5.6, 11.4); 4.39 (*dd*, 11.4)
Rhamnose		
1	5.07 (*d*, 1)	5.03 (*d*, 1)
2	3.73 (*dd*, 1, 3)	3.68 (*dd*, 1, 2.9)
3	3.33 (*dd*, 3, 9.5)	3.26 (*dd*, 2.9, 9.4)
4	3.14 (*dd*, 9.5, 9.5)	3.09 (*dd*, 9.4, 9.4)
5	3.40 (*m*)	3.35 (*m*)
6	0.99 (*d*, 6)	0.95 (*d*, 6)

Finally, the structure of **12** was established by comparison with **11** from its ^{1}H and ^{13}C NMR data. The ^{1}H NMR spectrum clearly shows the presence of two caffeoyl moieties close together and a β-(3,4-dihydroxyphenyl) ethoxy group. It displays also the signals due to the anomeric protons of two glucose and one rhamnose moieties as doublets at 4.34 ($J = 7.6$ Hz), 4.24 ($J = 7.6$ Hz) and 5.03 ppm ($J = 1$ Hz) respectively. The proton signals were assigned from a 2D homonuclear spectrum. The results are similar to those of **11** (Table 4) except for the presence of a new caffeoyl group and the downfield shift of H-$6_{G'}$ by *ca* 0.7 ppm. These data are consistent with acylation by a caffeoyl moiety of –OH at C-6 of the outer glucose G′. Thus **12** is β-(3,4-dihydroxyphenyl) ethyl-*O*-α-L-rhamnopyranosyl (1→3)(6-*O*-caffeoyl-β-D-glucopyranosyl)(1→6)-4-*O*-caffeoyl-β-D-glucopyranoside(6-*O*-caffeoyl-echinacoside). This structure was confirmed by ^{13}C NMR spectrometry (Table 3). The ^{13}C resonances of **11** and **12** are similar except at C-5 and C-6 of the glucose moiety G′. The signals attributable to C-5 and C-6 are shifted by −2.5 and 2.55 ppm respectively, due to acylation at C-6.

Table 5. Distribution of caffeoyl derivatives in the different parts of *Echinacea pallida*

	1	2	3	4	5	6	7	8	9	10	11	12
Flowers	++	++	++	+++	?	++	—	—	++	++	++	—
Leaves	++	+	+	+++	++	++	++	++	+	+	+	—
Roots	++	+	+	+++	?	++	—	—	+	+	+++	++

+: Traces; = ++: minor product; +++: major product.

Such results are in good agreement with previous results [30]. Finally FAB-MS was employed for determination and confirmation of structural features. The mass spectrum of **12** showed a $[M-H]^-$ signal at *m/z* 947 which corresponds to the molecular formula $C_{44}O_{23}H_{52}$. Two weak peaks at *m/z* 785 and *m/z* 623 were observed and can be attributed to loss of one and two caffeoyl groups respectively.

Although no exact quantitative analysis has been effected, it was possible from isolation and HPLC estimation to distinguish the major products (+++) from the products present in minor (++) and trace (+) amounts (Table 5). Except for **7**, **8** and **12**, all the identified compounds were extracted from dried flowers. Compounds **7** and **8** were isolated from the leaves and **12** was only detected in the roots. Chicoric acid **4** is the most important product in flowers and leaves. The flowers are characterized by the presence of **2** and **3** (in ratio *ca* 5:1) with **9** and **10**, also in appreciable amounts. Echinacoside **11** was detected for the first time in the flowers. The leaves principally contain chicoric acid, caftaric acid and the compounds **7** and **8** as minor components. The latter two were not found elsewhere in the plant. Compounds **2** and **3** were detected as traces upon HPLC analysis. In dried roots, the two most important components are echinacoside and chicoric acid. A minor amount of 6-*O*-caffeoylechinacoside was also found; this product seems to be characteristic of the roots. All these results are in good agreement with the chromatographic analysis (TLC, HPLC) from the different organs of fresh *E. pallida*.

Among these 12 products, only chicoric, chlorogenic and caftaric acids were identified by HPLC in dried *E. purpurea*. Unlike *E. pallida*, *E. purpurea* did not show marked differences in the distribution of products in the different organs. Chicoric acid was the major caffeoyl derivative. Caftaric acid was found in substantial quantities but chlorogenic acid was only present in small amount. Some less polar products were also detected as minor components but no further quantitative isolation was undertaken to identify them.

We have studied the effects of the two major products isolated from *E. pallida*, chicoric acid and echinacoside, on the yield reduction of VSV (Vesicular Stomatitis Virus) in mouse L-929 cells. We chose this assay system in order to compare our results with those reported by Wacker and Hilbig [7] on antiviral activity of whole plant extracts from *E. purpurea*. In order to discriminate between antiviral and toxic effects on host cells, we also studied the influence of two compounds on DNA metabolism and growth of freshly seeded cells. We included caffeic acid in our studies, since in a previous report, pronounced antiviral activity was attributed to this compound [25]. Our results are summarized in Table 6. Dose dependent antiviral activity was observed with all three products but was accompanied by marked inhibitory effects on cell growth and DNA metabolism. Caffeic acid proved to be highly toxic at 500 μg/ml. Therefore, we determined the therapeutic ratio (TR = 50% cytotoxic dose/minimum effective dose) according to Tsuchiya *et al.* (31). A TR value of 6 was obtained for all three substances, thus, allowing us to consider them as antiviral agents. It is unclear whether the observed antiviral effect of the drugs is due to inhibition of virus replication in infected cells alone or also to partial inactivation of released virus particles, since we found that incubation of VSV with 62.5 μg/ml of caffeic acid or 125 μg/ml of chicoric acid for four hr reduced infectivity of VSV by more than 50% (data not shown). In contrast, we, unlike Wacker and Hilbig, were not able to detect any antiviral effect when we treated L-929 cells with the drugs prior to VSV infection. This may indicate that there are additional antiviral compounds in *E. purpurea*. A dose response analysis of the antiviral and cell growth inhibitory effect of the three drugs revealed similar activities when molar relationships were considered (Table 6). As chicoric acid contains two caffeic acid moieties, this suggests that it produces its effects as an intact molecule or as a derivative and not as two molecules of caffeic acid following hydrolysis in the culture medium. In addition, we showed by TLC that no hydrolysis of chicoric acid occurred after 24 hr of incubation at 37° in the culture medium.

EXPERIMENTAL

General ^{1}H NMR data were determined at 400 MHz (locked to the deuterium resonance of the solvent CD_3OD or DMSO-d_6). A 45° pulse width at 3.75 interval was employed. Decoupling was performed using a homo-gated coupling unit. The protonated part of the solvent was used as int. standard (methanol: δ = 3.34 ppm, DMSO: δ = 2.50 ppm relatively to TMS). ^{13}C NMR spectra were measured at 100.6 MHz. ^{13}C shifts of the deuterated solvent was used as internal standard (methanol: δ = 49.9 ppm, DMSO: δ = 35.6 ppm relative to TMS). FAB mass spectra were recorded in thioglycerol using 8 KeV Xenon atoms (1 mA). SIMS were measured using cesium ions at 4 keV (1.2 mA). Secondly ion ionization was performed at 7 keV. HPLC analysis were performed using two pumps Milton Roy (ConstaMetric I and III), a Rheodyne 7510 injector and an UV detector at 328 nm (Spectromonitor D).

Plant material. The extraction and isolation of the different compounds were carried out on *Echinacea pallida* Nutt. cultivated and collected in Germany in 1983 (Bayerische Landesanstalt für Bodenkultur and Planzenbau, Sachgebiet PZ 3.3. Vöttingerstrasse 38, D-8050 Freising) and dried at 40°. An herbarium specimen is deposited at the University of Heidelberg. Fresh *E. pallida* were collected in the botanical garden of the University of Heidelberg.

Table 6. Antiviral and growth inhibitory effects on mouse L-229 cells of compounds isolated from *Echinacea pallida*

Concentration μg/ml	Concentration mmol/ml	VSV titre* (log PFU)	^{3}H-thymidine incorporation % of control	Cell count per culture % of control
Chicoric acid				
1000	2.11	6.13	36	20
500	1.05	6.50	49	48
250	0.52	7.00	60	59
125	0.26	7.30	69	70
62.5	0.13	7.42	90	80
31.25	0.065	7.68	100	92
15.6	0.033	7.8	100	97
Echinacoside				
1000	1.27	6.30	16	
500	0.64	6.60	38	
250	0.32	7.00	57	
125	0.16	7.20	82	nd
62.5	0.08	7.40	95	
31.25	0.04	7.62	100	
15.6	0.02	7.80	100	
Caffeic acid				
1000	5.55	2	1	1
500	2.78	2	1	1
250	1.39	6.30	40	28
125	0.69	6.85	71	71
62.5	0.35	7.24	80	87
31.25	0.17	7.45	88	91
15.6	0.09	7.63	93	95

* Results are presented as mean values of three individual experiments. VSV titre (log PFU) in untreated control cultures of L-929 cells was 7.82
nd: Not determined.

Extraction and isolation. Dried and finely powdered plant material (100 g) was extracted with MeOH–H_2O (4:1) (2 × 700 ml) at room temp. for 12 hr. The extracts were combined and MeOH evapd (30°, under red pres.). The aq. solution was successively extracted with petrol bp 40–60° (3 × 200 ml) and $CHCl_3$ (4 × 200 ml). After acidification to pH 3 with 2N HCl, the aqueous solution was further extracted with EtOAc (4 × 200 ml) and finally with *n*-BuOH (3 × 200 ml). The caffeoyl derivatives were isolated from the EtOAc extract (A) and the *n*-BuOH extract (B).

Each extract was concentrated and fractionated over Sephadex LH 20 (column: 35 × 4 cm) using MeOH–H_2O (4:1). The different fractions were checked by TLC. Precoated silica gel plates-(F_{254}, 0.25 mm, Merck) were used for the separation of the less polar products **1**–**10**, with EtOAc–H_2O–HCOOH mixture (10:9:1, upper phase) as eluting solvent (TLC, system I). Cellulose F precoated plates (Merck) were used to check more polar products such as **11** and **12** with *n*-BuOH–HOAc–H_2O (BAW, 4:1:5 upper phase) as mobile phase (TLC system II). Spots were detected by UV fluorescence (254 nm). The caffeoyl derivatives were characterized, as bright yellow spots by spraying with Naturstoffreagenz A followed by UV irradiation (366 nm).

The fractions from Sephadex LH 20 were further chromatographed over silica gel (Kieselgel MN 60, Macherey Nagel), Cellulose (MN 100) or polyamide (Woelm). The different products were finally purified on Sephadex LH 20 (MeOH–H_2O, 4:1) or with prep HPLC (Lichrospher RP 18, 7 μm, 250 × 10 mm; H_2O–MeOH–AcOH gradient).

Compound **1**. **1** was isolated from flowers (Extract A) as a white amorphous powder. After Sephadex partition, **1** was purified over Kieselgel eluting with EtOAc–MeOH (4:1) followed by an ascending concn of MeOH. TLC, system I: R_f 0.21. ^{1}H NMR spectrum (CD_3OD, 400 MHz), see Table 1.

Compounds **2** *and* **3**. These two compounds were isolated from flowers (Extract A). The fraction from Sephadex LH 20 containing **2** and **3** together was chromatographed over Kieselgel using EtOAc–MeOH–HCOOH (94.3:5:0.7) as mobile phase. **2** and **3** were successively isolated as pure amorphous white powders. TLC, system I: **2**: R_f 0.60; **3**: R_f 0.38. ^{1}H NMR spectrum (CD_3OD, 400 MHz) see Table 1.

Compound **4**. Chicoric acid was obtained as a powder from Sephadex LH 20 fractionating (MeOH–H_2O, 4:1). TLC, system I: R_f 0.55. ^{1}H NMR (DMSO-d_6, 2 drops of TFA): 7.56 (1H, *d*, *J* = 15.9 Hz, H-7′), 7.10 (1H, *d*, *J* = 2 Hz, H-2′), 7.07 (1H, *dd*, *J* = 2, 8, Hz H-6′), 6.80 (1H, *d*, *J* = 8 Hz, H-5′), 6.36 (1H, *d*, *J* = 15.9 Hz, H-8′), 5.67(1H, *s*, H-2). ^{13}C NMR spectrum (CD_3OD), see Table 2.

Compound **5**. **5** was isolated from leaves (extract A) upon prep HPLC (Lichrospher RP 18, 7 μm, 250 × 10 mm; H_2O–MeOH–HOAc, 77:8:15, 5 ml/mn; R_t 16.56 min). ^{1}H NMR (DMSO-d_6, 2 drops TFA): 7.61/7.55 (1H each, *d*, *J* = 16 Hz, H-7′), 7.38/7.10 (1H each, *d*, *J* = 2 Hz, H-2′), 7.12/7.03 (1H each, *dd*, *J* = 2, 8 Hz, H-6′), 6.80/6.77 (1H each, *d*, *J* = 8 Hz, H-5′), 6.59/6.32 (1H each, *d*, *J* = 16 Hz, H-8′) 5.70 (2H, *s*, H-2, H-3), 3.82 (3H, *s*, –OMe)

Compound **6**. **6** was isolated from roots (extract A, Sephadex LH 20) and further purified on cellulose using EtOAc–MeOH–HCOOH (100:15:1). Crystals. TLC, system I: R_f 0.16. ^{1}H NMR (DMSO-d_6, 2 drops TFA): 7.59 (1H, *d*, *J* = 15.9 Hz, H-7]′), 7.08 (1H, *d*, *J* = 1.8 Hz, H-2′), 7.04 (1H, *dd*, *J* = 1.8 and 8.2 Hz, H-6′), 6.81 (1H, *d*, *J* = 8.2 Hz, H-5′), 6.28 (1H, *d*, *J* = 15.9 Hz, H-8′), 5.34 (1H, *d*, *J* = 2.4 Hz, H-2), 4.57 (1H, *d*, *J*

= 2.4 Hz, H-3). ^{13}C NMR (CD_3OD), see Table 2.

Compound **7**. **7** was isolated in amorphous form from leaves (extract A) upon successive Sephadex LH 20 (MeOH–H_2O, 4:1. TLC, system I: R_f 0.46. ^{1}H NMR (DMSO-d_6, two drops TFA): 7.55/7.53 (1H, each, *d*, *J* = 16 Hz, H-7′, H-7″), 7.12 (1H, *d*, *J* = 2 Hz, H-2′), 7.01 (1H, *dd*, *J* = 2, 8 Hz, H-6′), 7.05/7.02 (1H each, *d*, *J* = 1.6 Hz, H-2″, H-6″), 6.77 (1H, *d*, *J* = 8 Hz, H-5′), 6.57 (1H, *d*, *J* = 2 Hz, H-2‴), 6.56 (1H, *d*, *J* = 8 Hz, H-5‴), 6.42 (1H, *dd*, *J* = 2, 8 Hz, H-6‴), 6.36/6.33 (1H each, *d*, *J* = 16 Hz, H-8′, H-8″), 5.70 (2H, *br s*, H-2, H-3), 4.11 (1H, *dd*, *J* = 8.0, 7.2, H-8‴), 3.07 (1H, *dd*, *J* = 8, 14 Hz, H-7‴), 2.79 (1H, *dd*, *J* = 7.2, 14 Hz, H-7‴). ^{13}C NMR (CD_3OD), see Table 2. SIMS (negative ion) *m/z* (rel. int.): 653 (4), 491 (2), 397 (2), 385 (4), 359 (7), 325 (3), 315 (5), 311 (8), 297 (6), 271 (6) 251 (4), 237 (6), 219 (5), 205 (8), 201 (7), 193 (55), 179 (30), 161 (25), 149 (54), 135 (30), 121 (18), 113 (30), 105 (100), 87 (59), 71 (57).

Compound **8**. **8** was obtained in amorphous form from leaves (Extract A) upon Sephadex LH 20 separation (MeOH–H_2O, 4:1). TLC, system I: R_f 0.34. ^{1}H NMR (DMSO-d_6, 2 drops of TFA): 7.54 (1H, *d*, *J* = 15.8 Hz, H-7″), 7.09/7.01 (1H each, *d*, *J* = 1.6 Hz, H-2″, H-6″), 6.58 (1H, *d*, *J* = 1.9 Hz, H-2‴), 6.52 (1H, *d*, *J* = 8 Hz, H-5‴), 6.42 (1H, *dd*, *J* = 1.9, 8 Hz, H-6‴), 6.34 (1H, *d*, *J* = 15.8 Hz, H-8″), 5.66 (1H, *s*, H-2), 4.11 (1H, *dd*, *J* = 7.2, 7.8 Hz, H-8‴), 3.08 (1H, *dd*, *J* = 13.8, 7.8 Hz, H-7‴), 2.79 (1H, *dd*, *J* = 7.2, 13.8 Hz, H-7‴). ^{13}C NMR (CD_3OD), see Table 2.

Compound **9**. **9** was isolated from flowers (extracts A and B). After fractionating over Sephadex LH 20, **9** was purified over Kieselgel eluting with EtOAc followed by ascending concns of MeOH and recovered as an amorphous powder. TLC, system I: R_f 0.48. ^{1}H NMR (DMSO-d_6, 2 drops TFA): caffeoyl moiety: 7.49 (1H, *d*, *J* = 15.8 Hz, H-7), 7.05 (1H, *d*, *J* = 1.9 Hz, H-2), 6.98 (1H, *dd*, *J* = 1.9, 8.2 Hz, H-6), 6.77 (1H, *d*, *J* = 8.2 Hz, H-5), 6.24 (1H, *d*, *J* = 15.9 Hz, H-8); 3,4-dihydroxy-β-phenethyl alcohol moiety: 6.64 (1H, *d*, *J* = 2 Hz, H-2), 6.63 (1H, *d*, *J* = 8 Hz, H-5), 6.49 (1H, *dd*, *J* = 2, 8 Hz, H-6), 3.85 (1H, *dt*, *J* = 9.6, 7.3 Hz, H-8), 3.7–3.6 (1H, *m*, H-8), 2.69 (2H, *dd*, *J* = 7.3 Hz, H-7); glucose moiety: 4.71 (1H, *dd*, *J* = 9.8, 9.4, H-4), 4.33 (1H, *d*, *J* = 7.8 Hz, H-1), 4.10 (1H, *dd*, *J* = 12.3, 6.2 Hz, H-6), 4.05 (1H, *dd*, *J* = 12.3, 3.0 Hz, H-6), 3.6–3.7 (1H, *m*, H-5), 3.49 (1H, *dd*, *J* = 9.4, 9.1 Hz, H-3), 3.13 (1H, *dd*, *J* = 9.1, 7.8 Hz, H-2). ^{13}C NMR (DMSO), see Table 3.

Compound **10**. **10** was isolated from flowers (Extract B) and obtained pure pt upon successive Sephadex LH 20 column (MeOH–H_2O, 4:1). TLC, System I: R_f 0.27; TLC, system II: R_f 0.50. ^{1}H and ^{13}C NMR were in agreement with ref. [27].

Compound **11**. **11** was isolated from flowers and from roots (Extract B). After Sephadex preliminary separation, **11** was purified over polyamide eluting with H_2O followed by ascending concentration of MeOH and obtained as a pale beige powder. TLC, system II: R_f 0.27. ^{1}H and ^{13}C NMR reported as comparative data (Tables 4 and 3) were in agreement with ref. [20].

Compound **12**. **12** was extracted from roots (Extracts A and B) and isolated pure pt over cellulose eluting with EtOAc–MeOH–H_2O (20:3:2). TLC, system I: R_f 0.12; TLC, system II: R_f 0.37. ^{1}H and ^{13}C NMR are reported in Tables 4 and 3. FABMS (Negative ions) *m/z*: 947 $[M-H]^-$, 785 and 623 (each about 16% of mass peak).

BIOLOGICAL TESTS

Mouse L-929 cells were cultured in Dulbecco's modified Minimal Essential Medium (DMEM) supplemented with 5% calf serum (FCS). Cells were grown to confluency in 24 well plates (Becton and Dickinson, Heidelberg, F.R.G.) at 37° in a 5% CO_2 and H_2O saturated atmosphere. They were infected with Vesicular Stomatitis Virus (VSV, strain Indiana, obtained from the American Type Culture Collection) at a multiplicity of infection of 0.2. Following 1–2 hr of incubation at 37°, non-attached virus was removed and cell monolayers were incubated with two-fold dilutions of chicoric acid or echinacoside in DMEM plus 5% FCS buffered to pH 7.2. 12 hr later the culture plates were frozen at −70° and titres of infectious virus were measured as plague forming units (PFU) in a standard viral plaque assay.

The effect of drug on proliferation of cells in culture was measured by ^{3}H-thymidine uptake. Confluent cultures of L-929 cells in 96 well cultures (Becton and Dickinson, Heidelberg, F.R.G.) were incubated with twofold dilutions of the drugs for 24 hr, pulsed for 4 hr with 10 μCi per ml of ^{3}H-thymidine and harvested into cellulose filters using Skatron multiple cell culture harvestor (Skatron, Lierbyen, Norway). The filters were counted in a Packard scintillation counter in 3 ml of Rotiszint (Roth, Karlsruhe, F.R.G.).

Influence of the drugs on replication of L-929 cells was studied by growing freshly seeded cells in petri dishes in the presence of two-fold dilutions of the drugs in culture medium. 24 hr later, the cells were trypsinized and numbers of viable cells per culture were determined microscopically by trypan exclusion.

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REFERENCES

1. Pohl, P. (1969) *Med. Klin.* **64**, 1546.
2. Becker, H. (1982) *Dtsch. Apoth. Ztg* **122**, 2320.
3. Funke, H. (1983) *Z. Phytother.* **5**, 650.
4. Orinda, D., Diederich, J. and Wacker, A. (1973) *Arzneim. Forsch.* **23**, 1.
5. Eilmes, H. G. (1976) Diss. Frankfurt/M.
6. May, G. and Willuhn, G. (1978) *Arzneim. Forsch.* **28**, 1.
7. Wacker, A. and Hilbig, W. (1978) *Planta Med.* **33**, 89.
8. Wagner, H. and Proksch, A. (1981) *Z. Angew. Phytother.* **2**, 166.
9. Schimmel, K. C. and Werner, G. T. (1981) *Ther. Gegenw.* **120**, 1065.
10. Bauer, R., Khan, I. A., Wray, V. and Wagner, H. (1987) *Phytochemistry* **26**, 1198.
11. Bauer, R., Wray, V. and Wagner, H. (1987) *Pharmaceutisch Weekblad, Scientific Edition* **9**, 220.
12. Stoll, A., Renz, J. and Brack, A. (1950) *Helv. Chim. Acta*, 33, 473.
13. Jacobson, M. (1954) *Science* **120**, 1028.
14. Bohlmann, F. and Grenz, M. (1966) *Chem. Ber.* **99**, 3197.
15. Schulte, K. E., Rücker, C. and Perlick, J. (1967) *Arzneim. Forsch.* **17**, 825.
16. Jacobson, M. (1967) *J. Org. Chem.* **32**, 1646.
17. Voaden, D. J. and Jacobson, M. (1972) *J. Med. Chem.* **15**, 619.
18. Jacobson, M., Redfern, R. and Milis, G. (1975) *Lloydia* **38**, 473.
19. Verelis, C. D. and Becker, H. (1977) *Planta Med.* **31**, 288.
20. Becker, H., Hsieh, W. C., Wylde, R., Laffite, C. and Andary, C. (1982) *Z. Naturforsch.* **37c**, 351.
21. Malonga-Makosi, J. P. (1983) Diss. Heidelberg.
22. Becker, H. and Hsieh, W. C. (1985) *Z. Naturforsch.* **40c**, 585.

23. Bauer, R., Kahn, I. A. and Wagner, H. (1986) *Dtsch Apoth. Ztg* **126**, 1.
24. Wagner, H., Stuppner, H., Puhlmann, J., Jurcic, K., Zenk, M. H. and Lohmann-Matthes, M. L. (1986) *Planta Med.* 428.
25. König, B. and Dustmann, J. H. (1985) *Naturwissenschaften* **72**, 659.
26. Scarpati, M. L. S. and Oriente, G. (1958) *Tetrahedron* **4**, 43.
27. Andary, C., Wylde, R., Laffite, C., Privat, G. and Winternitz, F. (1982) *Phytochemistry* **21**, 1123.
28. Lahloub, M. F., Gross, G. A., Sticher, O., Winkler, T. and Schulten, H. R. (1986) *Planta Med.* 352.
29. Shimomura, H., Sashida, Y. and Ogawa, K. (1987) *Phytochemistry* **26**, 1981.
30. Shimomura, H., Sashida, Y. and Adachi, T. (1987) *Phytochemistry* **26**, 249.
31. Tsuchiya, Y., Shimizu, M., Hiyama, Y., Itah, K., Hashimoto, Y., Nakayama, N., Horie, T. and Morita, N. (1985) *Chem. Pharm. Bull* **33**, 3881.