

SUPPLEMENTAL Tables and Figures

Chemical Characterization of Select *Echinacea Purpurea* Extract Materials

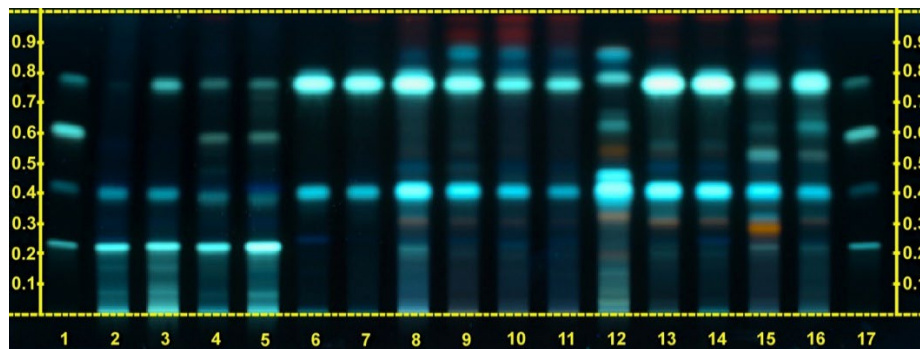
Three samples (EC 22, 23, and 24) were selected for chemical characterization. In some analyses *E. purpurea* root extract XRM (EC15) and *E. purpurea* root extract, which has been used in previous toxicity studies (EC13) were included for comparison.

Authentication of EC 22, 23, and 24 by high performance-thin layer chromatography (HPTLC)

HPTLC analysis was conducted by Alkemist Labs (Garden Grove, CA) using laboratory protocols. Briefly, samples (6 μ L of extract), species reference samples (6 μ L of extract), and a constituent standard mix (3 μ L) were spotted on a Silica gel 60, F254, HP-TLC plate, developed using ethyl acetate:acetic acid:formic acid:water (10/1.1/1.1/1.2), treated with natural product reagent and polyethylene glycol, and visualized at 366 nm. Echinacea samples EC 22, 23, and 24 were analyzed along with EC 15 (*E. purpurea* root extract XRM) (lane 8) by HPTLC along with Alkemist Labs standard reference material for Echinacea species.

The pattern and retention factors (Rf) for bands of materials EC22 (lane 10), EC23 (lane 11), and EC24 (lane 9) were similar to each other and that of *E. purpurea* root extract XRM, EC15 (lane 8) but was different from the pattern for reference materials for other Echinacea species (*E. pallida*, lanes 2 and 3; *E. angustifolia*, lanes 4 and 5) (Supplemental Figure 1). Although the constituent pattern of test materials is also similar to Alkemist Labs *E. purpurea* root reference material (lanes 6 and 7), some constituents were not visible likely due to levels of constituents being low. Bands correlating to Rfs \sim 0.77 (chichoric acid) and \sim 0.41 (caftaric acid) were similar to Rfs in test materials (lanes 9-11), *E. purpurea* root extract XRM (lane 8) and Alkemist Labs' reference for *E. purpurea* root (lanes 6-7). Overall, the HPTLC pattern of EC 22, 23, and 24 resembled *E. purpurea* and was different from that of *E. pallida* and *E. angustifolia*.

Supplemental Figure 1



Supplemental Figure 1. Chromatogram from HPTLC analysis. Sample key for only the relevant samples are given: Standard Mix (echinacoside Rf 0.23, caftaric acid Rf 0.41, cynarin Rf 0.60, chicoric acid Rf 0.77) = lanes 1 and 17; *E. pallida* root lab standard = lanes 2 and 3; *E.*

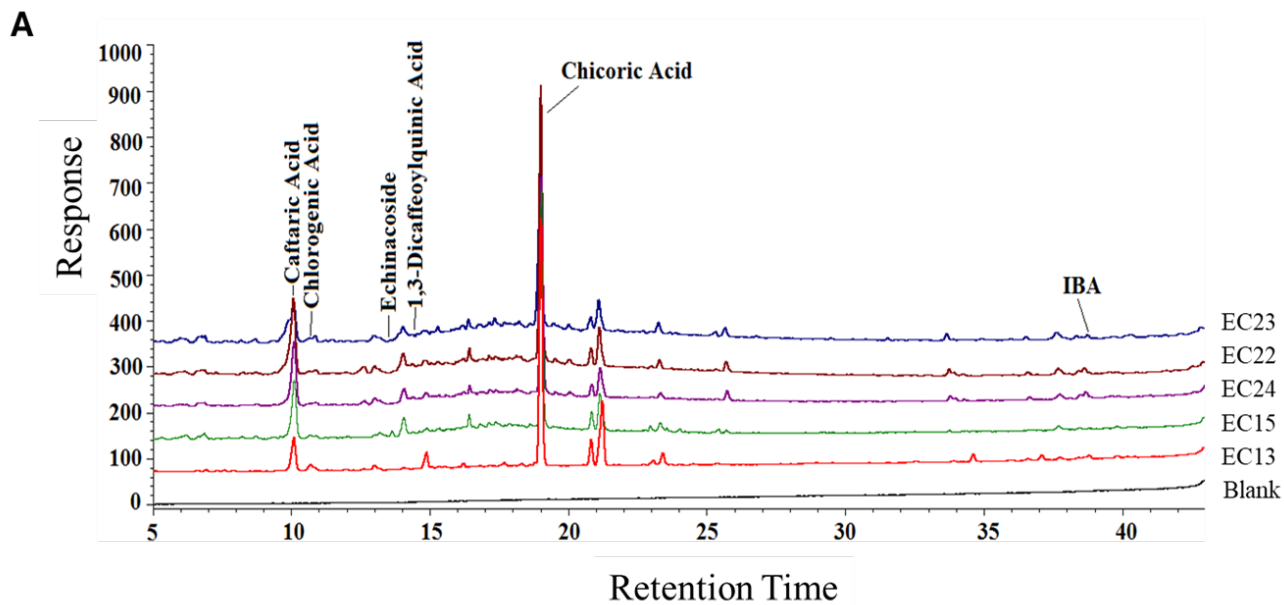
angustifolia root lab standard = lanes 4 and 5; *E. purpurea* root lab standard = lanes 6 and 7; *E. purpurea* aerial parts lab standard = lane 13; EC15 = lane 8; EC22 = lane 10; EC23 = lane 11; EC24 = lane 9.

Analysis of EC 22, 23, and 24 by high performance liquid chromatography coupled with charged aerosol detection (HPLC-CAD)

EC 22, 23, and 24 were analyzed by HPLC-CAD and corresponding chromatographic profiles are shown in Supplemental Figure 2A. A visual comparison showed that chromatographic profiles of lots EC22-24 were similar to lot EC13 (previously tested lot) and to the *E. purpurea* root extract reference material, XRM (EC15) further demonstrating the identity of EC22-24 materials as *E. purpurea*.

Constituent concentration in samples were estimated using a standard addition method using HPLC-CAD analysis as described previously (Waidyanatha et al. 2020). Briefly, the method involved spiking each of the sample with standards of constituents to be quantified at approximately 0 (unspiked), 1x, 2x, and 4x of the expected concentration based on preliminary analysis. From the data, a linear regression was generated which was subsequently used to calculate the concentration of analytes in unspiked samples. Next, constituent levels estimated are given in Supplemental Figure 2B. In general, constituent levels between EC 22, 23, and 24 were within 1- to 3-fold depending on the constituent with total estimated levels ranging from 5.58 to 8.30% (weight of constituent/weight of sample) and are suitable as *E. purpurea* root extract for *in vivo* testing.

Supplemental Figure 2

**B**

Constituent	EC13	EC15	EC22	EC23	EC24
Chicoric Acid	5.38	5.20	5.78	4.27	6.08
Caftaric Acid	0.70	1.34	2.21	1.23	2.17
1,3-Dicaffeoylquinic Acid	0.00380	0.00446	0.00312	0.00676	0.00248
Echinacoside	0.00950	0.0681	0.00336	0.00187	0.00994
IBA	0.0813	0.00696	0.0230	0.0606	0.0123
Chlorogenic Acid	0.141	0.039	0.018	0.012	0.030
Total	6.31	6.66	8.04	5.58	8.30

Supplemental Figure 2. Quantification and comparison of *Echinacea* constituents in EC22-24 to EC13 (previously tested *E. purpurea* root extract) and EC15 (*E. purpurea* root extract reference material). A) Representative HPLC-CAD chromatograms. B) Estimated constituent levels (% weight/weight) of echinacea markers.

Contaminants analysis

E. purpurea materials EC 22, 23, and 24 were analyzed along with EC15 (*E. purpurea* root extract reference material) to determine the presence/absence of contaminants to guide selection of a representative *E. purpurea* extract for *in vivo* testing. Analyses were conducted by Covance Laboratories (Madison, WI), <https://www.covance.com/>) with a panel that included 310 pesticides. Typical limits of quantitation (LOQ) were between 0.01 and 0.5 ppm. Only the analytes above the LOQ in at least one lot are shown in Supplemental Table 1. Aerobic plate count, which is used as an indicator of bacterial contamination ranged from 2600-42,000 CFU/g in the tested samples. The assays for *Salmonella*, *E. coli*, and *Staphylococcus enterotoxins* were negative in all 3 samples. Five of the heavy metals analyzed were above the LOQ except mercury in EC22. Overall, heavy metals were lowest in EC23. Of the 310 pesticides screened, most were below the LOQ of the assay. Overall, the data showed that contaminant levels were minimal in EC 22, 23, and 24.

Supplemental Table 1. Contaminant Analysis of Select *E. purpurea* root extract materials

Endpoint	EC13	EC22	EC23	EC24
Aerobic plate count (CFU/g)	<10	42000	2600	3800
<i>E. coli</i> (per 1 g)	<10	Absent	Absent	Absent
<i>Salmonella</i> (per 10 g)	Negative	Absent	Absent	Absent
<i>Staphylococcus enterotoxins</i> (per 25 g)	Negative	Negative	Negative	Negative
Aflatoxins (B1, B2, G1, G2)	<1.0	2.24 B1 <0.5 B2, G1, G2	<0.5	<0.5
Ochratoxin	<1.0	3.77	1.80	<1.0
Zearalenone	<10.0	<30	61.1	<30
Antimony	<10.0	38.6	11.4	21.9
Arsenic	129	223	127	267
Cadmium	12.9	21.2	19.1	17.1
Lead	36.4	195	78.9	82.3
Mercury	<10.0	<10.0	12.0	17.9
Acetamiprid	<0.01	0.23	<0.05	<0.05
Azoxystrobin	0.070			
Bifenthrin	<0.01	0.020	<0.05	<0.05
Boscalid	0.2			
Captan (Tetrahydrophalimide)	Not Analyzed	<0.2	<0.2	0.01
Carbaryl	<0.01	<0.05	<0.1	<0.05

Carbendazin	<0.01	0.16	0.18	<0.05
Chlorantraniliprole	<0.01	0.021	<0.05	0.021
Chlorpropham	0.057	<0.05	<0.05	<0.05
Chlorpyrifos	<0.01	0.21	0.042	<0.01
Difencoconazole	0.079	0.026	<0.05	<0.05
Epoxiconazole	0.011	<0.05	<0.05	<0.05
Myclobutanil	<0.01	0.019	<0.05	<0.05
Paclobutrazole	<0.01	0.030	<0.05	<0.05
Pendimethalin	0.091	<0.05	<0.05	<0.05
Pentachloroaniline	<0.01	0.026	<0.05	0.044
Phenylphenol, 2- (OPP)	0.016	<0.05	<0.05	<0.05
Procimidone	<0.01	0.51	<0.05	<0.05
Pyridaben	<0.01	0.022	<0.05	<0.05
Quintozene	<0.01	<0.01	<0.05	0.028
Tebuconazole	<0.01	0.032	<0.05	<0.05
Trifloxystrobin	0.28	<0.05	<0.05	<0.05

Comprehensive characterization of *E.purpurea* material for in vivo testing

Based on the chemical screening data, EC23 (lot 0905704; Gojira Fine Chemicals, LLC, Bedford Heights, OH) was selected for the *in vivo* studies. Lot 0905704 was received in 8 containers. The bulk material in multiple containers was homogenized by blending, repackaged, and assigned a lot number 02252019 and additional characterization was performed. The characterization approach and methodologies used were similar to those published previously in Waidyanatha et al 2020.

Briefly, weight loss on drying was performed to estimate moisture and/or solvent content and was 6.8% and average inorganic content, determined as ash, was 13.3 %. Water content (3.2%) and inorganic content (12.8%) was also determined by the second lab. Nutritional analyses were conducted by up to two labs; fat, 1.1-2.8%; total carbohydrate (simple and complex carbohydrates); 43.3-50.4%; protein, 7.3%.

Proton induced x-ray emission (PIXE) elemental analysis was performed. Potassium (4.6%) and chlorine (1.1%) were the two most abundant elements detected, and the summed percent of all of the elements analyzed was 7.3%.

Liquid chromatography with time of flight (TOF) tandem mass spectrometry (LC-MS/MS) was used to identify constituents in the lot, and the results are summarized in Supplemental Table 3. Eight compounds, which are known constituents of *E. purpurea*, were definitively identified using authentic standards (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, undec-2-ene-8,10-diynoic acid isobutylamide, dodec-2E-ene-8,10-diynoic acid isobutylamide, dodeca-2E,4E-dienoic acid isobutylamide, 4,5-dicaffeoylquinic acid, caftaric acid, chlorogenic acid, and chicoric acid). Ten others were tentatively identified based on exact mass matches with known *E. purpurea* constituents.

Supplemental Table 2. Constituents identified in *E. purpurea* lot 02252019 by TOF-MS using library match and exact mass (± 0.002 Da)^{a,b}

Constituent	Ref ^b	Species ^a	CAS	Formula	M.W.	Exact Mass M+H M-H	Standard Analyzed	m/z Found	RT (mins)	Library Match (yes/no)
Chicoric Acid	1	EP, EA, EL	6537-80-0	C ₂₂ H ₁₈ O ₁₂	474.371	475.0877 473.072	Yes	473.0717 473.0712	18.89 22.83	Yes
Caftaric Acid	1	EP, EA, EL	67879-58-7	C ₁₃ H ₁₂ O ₉	312.229	313.056 311.221	Yes	313.0553 311.0404 311.0405	9.84 9.75 11.50	Yes
Chlorogenic Acid	1	EP, EA, EL	327-97-9	C ₁₆ H ₁₈ O ₉	354.309	355.1029 353.0873	Yes	355.1027 353.0868	10.3	Yes
Caffeoyl feruloyl tartaric acid	5	EP, EL	Unknown	C ₂₃ H ₂₀ O ₁₂	488.398	489.1033 487.0877	No	487.0873	21.13	No
Diferuloyltartaric acid	5	EP	Unknown	C ₂₄ H ₂₂ O ₁₂	502.424	503.119 501.1033	No	501.1028 501.1059	23.40 23.38	No
Chicoric acid methyl ester	3	EP	Unknown	C ₂₃ H ₂₀ O ₁₂	488.398	489.1033 487.0877	No	487.0873	21.13	No
Feruloyltartaric acid	3	EP	1044-65-1	C ₁₄ H ₁₄ O ₉	326.256	327.0716 325.056	No	325.0558 325.0559 325.0559	14.44 15.64 13.49	No
2-caffeoyl-3- <i>p</i> -coumaroyl tartaric acid	3	EP	Unknown	C ₂₂ H ₁₈ O ₁₁	458.372	459.0927 457.0771	No	457.0765	20.83	No
1,3-Dicaffeoylquinic Acid (Cynarin Isomer)	1	EA (-EP)	30964-13-7	C ₂₅ H ₂₄ O ₁₂	516.451	517.1346 515.119	Yes	515.1176	14.11	Yes

1,5-Dicaffeoylquinic Acid (Cynarin Isomer)	1	EA (-EP)	19870-46-3	C ₂₅ H ₂₄ O ₁₂	516.451	517.1346	Yes	515.118	18.46	Yes
3,5-Dicaffeoylquinic Acid (Cynarin Isomer)	3	EL (-EP)	2450-53-5	C ₂₅ H ₂₄ O ₁₂	516.451	517.1346 515.119	Yes	515.1179	17.97	Yes
4,5-Dicaffeoylquinic Acid (Cynarin Isomer)	3	EL (-EP)	57378-72-0	C ₂₅ H ₂₄ O ₁₂	516.451	517.1346 515.119	Yes	515.1181	17.4	Yes
Undeca-2E,4Z-diene-8,10-diyonic acid isobutylamide	1	EP, EA	Unknown	C ₁₅ H ₁₉ NO	229.317	230.1545 228.1388	No	230.1539	32.05	Yes
Undeca-2Z,4E-diene-8,10-diyonic acid isobutylamide	1	EP, EA	13894-69-4	C ₁₅ H ₁₉ NO	229.317	230.1545 228.1388	No	230.1539	32.05	Yes
Undec-2E-ene-8,10-diyonic acid isobutylamide	4	EP, EA	99615-81-3	C ₁₅ H ₂₁ NO	231.333	232.1701 230.1545	Yes (Mixture of Isomers)	232.1698	33	Yes
Undec-2Z-ene-8,10-diyonic acid isobutylamide	4	EA	Unknown	C ₁₅ H ₂₁ NO	231.333	232.1701 230.1545	Yes (Mixture of Isomers)	232.1698	33	Yes
Undeca-2E,4Z-diene-8,10-diyonic acid 2-methylbutylamide	4	EP, EA	99615-79-9	C ₁₆ H ₂₁ NO	243.344	244.1701 242.1545	No	244.1693 244.1695 244.1695	35.17 34.53 33.99	No

Undeca-2Z,4E-diene-8,10-diynoic acid 2-methylbutylamide	4	EP, EA	1166938-46-0	C ₁₆ H ₂₁ NO	243.344	244.1701 242.1545	No	244.1693 244.1695 244.1695	35.17 34.53 33.99	No
Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	1	EP, EA	113817-69-9	C ₁₆ H ₂₁ NO	243.344	244.1701 242.1545	No	244.1693 244.1695 244.1695	35.17 34.53 33.99	No
Dodec-2E-ene-8,10-diynoic acid isobutylamide	4	EP	120727-29-9	C ₁₆ H ₂₃ NO	245.36	246.1858 244.1701	Yes	246.1852	35.13	Yes
Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide	1, 4	EP, EA	77448-63-6	C ₁₆ H ₂₅ NO	247.376	248.2014 248.1858	Yes (Mixture of Isomers)	248.2009	38.03	Yes
Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	1, 4	EP, EA	75917-90-7	C ₁₆ H ₂₅ NO	247.376	248.2014 248.1858	Yes (Mixture of Isomers)	248.2010	38.05	No
Dodeca-2E,4E,8Z-trienoic acid isobutylamide	1	EP	1166938-57-3	C ₁₆ H ₂₇ NO	249.392	250.2171 248.2014	No	250.2168 250.2167	40.58, 39.10	No
Dodeca-2E,4E-dienoic acid isobutylamide	1	EP	24738-51-0	C ₁₆ H ₂₉ NO	251.408	252.2327 250.2171	Yes	252.2324	44.14	Yes
Trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide	1	EP, EA	87797-74-8	C ₁₇ H ₂₃ NO	257.371	258.1858 256.1701	No	258.1852	35.97	No
Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	1	EP	117505-95-0	C ₁₇ H ₂₃ NO	257.371	258.1858 256.1701	No	258.1853	36.42	No

Dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	4	EP	Unknown	C ₁₇ H ₂₇ NO	261.402	262.2171 260.2014	No	262.2166	40.39	No
Pentadeca-2E,9Z-diene-12,14-dienoic acid isobutylamide	3	EL	Unknown	C ₁₉ H ₂₃ NO	285.424	286.2171 284.2014	No	286.2167	40.32	No
Glycine betaine	1	EP	107-43-7	C ₅ H ₁₁ NO ₂	117.146	118.0868 116.0712	Yes	118.0868	3.24	No
Nicotiflorin	2, 3	EP	17650-84-9	C ₂₇ H ₃₀ O ₁₅	594.518	595.1663 593.1506	No	593.1499 593.1496	17.14 16.81	No
Rutin	2, 3	EP	153-18-4	C ₂₇ H ₃₀ O ₁₆	610.518	611.1612 609.1456	No	609.1447	16.04	Yes

EL: *Echinacea pallida*, EA: *Echinacea angustifolia*, EP: *Echinacea purpurea*

^a Species if listed in literature. A "-" indicates absence in that species specifically noted. All of the isobutylamides are trace or absent in *Pallida*.

^b References:

1. American Herbal Pharmacopoeia (AHP) *Echinacea Purpurea* Root Monograph.
2. Kurkin, V.A., Akushskaya, A.S., Avdeeva, E.V., Velmyaikina, E.I., Daeva, E.D., Kadentsev, V.I. "Flavenoids from *Echinacea Purpurea*", *Russian Journal of Bioorganic Chemistry* 2011, 37:905-906
3. *Echinacea* - The genus *Echinacea*; Miller, S. C., Yu, He-ci, Eds; CRC Press: New York, 2004.
4. Spelman, K., Wetschler, M.H., Cech, N.B. "Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry *Echinacea purpurea* utilizing HPLC-ESI-MS", *J Pharm Biomed Anal.* 2009, 49: 1141-1149
5. Lu, Y., Li, J.Y., Li, M.L., Hu, X., Tan, J., Liu, Z.H. "Efficient Counter-current Chromatographic Isolation and Structural Identification of Two New Cinnamic Acids from *Echinacea Purpurea*", *Natural Product Communications* 2012, 7: 1353-1356

Selected constituents of *E. purpurea* and potential adulterants were quantitated by LC-MS/MS using a validated method (Waidyanatha et al. 2020) and corresponding data are shown in Supplemental Table 3 along with data for previously evaluated material (Waidyanatha et al. 2020). The higher percent of chicoric acid and non-detection of echinacoside support the lot as *E. purpurea* and not other Echinacea species. The % constituents detected were within 2-fold of the previously characterized test material. Also, total % constituents detected in EC23 was 1.80 and was similar to EC13, the previous tested material (2.08%). Constituents estimated by LC-MS/MS were lower than those estimated using HPLC-CAD (Supplemental Figure 2B) demonstrating the need for use of specific detectors such as mass spectrometry for quantitation of constituents in Echinacea.

Supplemental Table 3. Percent constituents in *E. purpurea* sample EC23 and comparison to EC13

Constituent	Percent (wt./wt.)	
	EC23	EC13 ^a
Chicoric acid	0.97	1.52
Chlorogenic acid	0.03	0.07
Caftaric acid	0.71	0.37
Echinacoside	ND	ND ^b
Dodeca-2(E),4(E), 8(Z),10(E/Z)-tetraenoic acid isobutylamide (DTA-IBA)	0.10	0.11
1,3-Dicaffeoylquinic acid (cynarin isomer)	ND	ND
<i>Total constituents</i>	<i>1.80</i>	<i>2.08</i>

^aData from Waidyanatha et al. 2020

^bND, not detected above the limit of detection.