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Synergistic Antioxidative Effects of Alkamides, Caffeic Acid Derivatives, and Polysaccharide Fractions from *Echinacea purpurea* on in Vitro Oxidation of Human Low-Density Lipoproteins

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Preparations of *Echinacea* are widely used as alternative remedies to prevent the common cold and infections in the upper respiratory tract. After extraction, fractionation, and isolation, the antioxidant activity of three extracts, one alkamide fraction, four polysaccharide-containing fractions, and three caffeic acid derivatives from *Echinacea purpurea* root was evaluated by measuring their inhibition of in vitro Cu(II)-catalyzed oxidation of human low-density lipoprotein (LDL). The antioxidant activities of the isolated caffeic acid derivatives were compared to those of echinacoside, caffeic acid, and rosmarinic acid for reference. The order of antioxidant activity of the tested substances was cichoric acid > echinacoside ≥ derivative II ≥ caffeic acid ≥ rosmarinic acid > derivative I. Among the extracts the 80% aqueous ethanolic extract exhibited a 10 times longer lag phase prolongation (LPP) than the 50% ethanolic extract, which in turn exhibited a longer LPP than the water extract. Following ion-exchange chromatography of the water extract, the majority of its antioxidant activity was found in the latest eluted fraction (H_2O-acidic 3). The antioxidant activity of the tested *Echinacea* extracts, fractions, and isolated compounds was dose dependent. Synergistic antioxidant effects of *Echinacea* constituents were found when cichoric acid (major caffeic acid derivative in *E. purpurea*) or echinacoside (major caffeic acid derivative in *Echinacea pallida* and *Echinacea angustifolia*) were combined with a natural mixture of alkamides and/or a water extract containing the high molecular weight compounds. This contributes to the hypothesis that the physiologically beneficial effects of *Echinacea* are exerted by the multitude of constituents present in the preparations.

KEYWORDS: ***Echinacea purpurea*; phenylpropanoids; cichoric acid; echinacoside; alkamides; alkylamides; caffeic acid derivatives; polysaccharide fractions; antioxidant; LDL oxidation; synergy**

INTRODUCTION

Preparations of *Echinacea* species are widely used as immunostimulants to prevent/cure the common cold and infections in the upper respiratory tract. In Europe *Echinacea* was the 10th most important medicinal plant sold in 1998, with annual sales of 120 million U.S.$ (*1*, *2*). In the United States *Echinacea* ranks as number three among the top-selling herbal dietary supplements, and with a value of annual sales of more than 32 million U.S.$ *Echinacea* products account for >10% of the sales in U.S. conventional food, drug, and mass market retail stores (*3*). In Australia *Echinacea* is a market leader, too (*2*, *4*), and *Echinacea* is experiencing increasing attention in North Africa, South America, and China (*2*). Three species of *Echinacea* are now used worldwide for medicinal preparations: *E. purpurea*, *E. pallida*, and *E. angustifolia* [= *E. pallida* ssp. *angustifolia* (*5*, *6*)] (*2*).

Investigations of antimicrobial, antiviral, antioxidant, and general immunostimulating effects have been in focus as mechanisms explaining the putative effects of *Echinacea* preparations on upper respiratory tract infections (*7*, *8*). In the search for the active principles in *Echinacea*, four groups of substances are referred to as the most important: phenylpropanoids, alkamides, polysaccharides, and glycoproteins (*9*). Phenylpropanoids are phenolics and known to be good antioxidants, whereas little is known about the antioxidant activity of the other important constituents of *Echinacea*. The major phenylpropanoids are caffeic acid derivatives; for example, in

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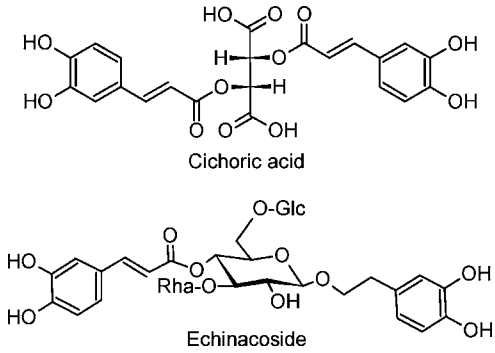

Figure 1. Chemical structures of cichoric acid (major caffeic acid derivative in *E. purpurea*) and echinacoside (major caffeic acid derivative in *E. angustifolia* and *E. pallida*).

E. purpurea cichoric acid (2*R*,3*R*-dicaffeoyl tartaric acid) dominates (**Figure 1**), whereas in both *E. pallida* and *E. angustifolia* echinacoside is the major caffeic acid derivative (**Figure 1**) (*10*). Among the known minor constituents in *Echinacea*, the flavonoids and anthocyanins are known to have antioxidant properties, but a systematic study of the antioxidant activity of the mixture of these substances found in *Echinacea* is not available (*11*). Nevertheless, the high level of caffeic acid derivatives has paved the way for the hypothesis that some of the physiological effects of *Echinacea* may be related to the antioxidative activity of these compounds (*12, 13*). Free radicals in high amounts are deleterious to the immune function, but at the same time the generation of free radicals by, for example, neutrofils is used by the immune system to kill invading pathogens; hence, antioxidants can increase immune responses by balancing the amount of free radicals and thereby preserving immune cells (*14*).

The previous literature on *Echinacea* and antioxidant activity can be conveniently divided into three groups: (1) screenings in which *Echinacea* extracts are compared to extracts of other medicinal or aromatic plants, (2) comparisons of free radical scavenging activity of various extracts of the commercial *Echinacea* species, and (3) comparisons of the antioxidant activity in different antioxidant test model systems of some of the individual phenolics found in *Echinacea*.

The antioxidant and radical scavenging activities of extracts of *Echinacea* have thus been compared to those of extracts of other medicinal, aromatic, and food plants using various methods: β-carotene−linoleate (*13*, *15*, *16*), scavenging of DPPH$^{\bullet}$ (*13*, *17*), scavenging of ABTS$^{\bullet+}$ (*17*, *18*), ferric reducing ability of plasma (*12*), and scavenging of hydroxyl radicals investigated with electron paramagnetic resonance (EPR) (*13*). Generally, the tested *E. purpurea* extracts showed medium to low activity compared to the other investigated medicinal and aromatic plants.

Comparisons of the antioxidant activities of extracts of the three commercially grown *Echinacea* species determined the order of radical scavenging activity of root extracts to be *E. purpurea* > *E. pallida* > *E. angustifolia* in the DPPH$^{\bullet}$ according to Pellati et al. (*19*) and in the ABTS$^{\bullet+}$ assay according to Sloley et al. (*20*), whereas Hu and Kitts (*21*) found that their methanolic root extract of *E. pallida* had the highest ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging activities and the highest reducing power and gave the best protection against peroxyl radical induced peroxidation of liposomes compared to *E. purpurea* and *E. angustifolia*. The extracts of all three species of *Echinacea* were found to exhibit similar protection against Cu^{2+}-induced oxidation of human low-density lipoproteins (LDL) (*21*).

The radical scavenging activity of some of the major phenylpropanoids found in *Echinacea* species has been determined using the DPPH$^{\bullet}$ assay (*19*), the ABTS$^{\bullet+}$ assay (*20*), and the Briggs−Rauscher method (*22*), but to our knowledge not in the LDL $CuSO_4$ assay.

To model the structure−activity relationship between phenolics, different benzoic and cinnamic acid derivatives have been tested in the LDL $CuSO_4$ assay by several authors (*23*−*25*), who found that the antioxidative properties largely depend on the substitution in the aromatic ring: "the antioxidant activity improves as the number of hydroxyl or methoxyl groups increases, and particularly the presence of the *o*-dihydroxy group in the phenolic ring, as in caffeic acid, consistently enhances antioxidant activity" (*23*).

Previous work has focused on the antioxidant activity of extracts or individual caffeic acid derivatives. Recently, Thulin and Thygesen (*26*, *27*) found indications of a synergistic effect when testing a combination of cichoric acid and an alkamide mixture in an antioxidant assay measuring the oxygen consumption in a peroxidating lipid emulsion. The antioxidant activity of cichoric acid alone was found to be comparable with that of rosmarinic acid; the alkamide mixture alone showed prooxidative or no antioxidant activity, whereas a combination of cichoric acid with the alkamide mixture resulted in higher antioxidant activity of the combination than for cichoric acid alone. This finding is very interesting because the observed activity of preparations of *Echinacea* often has been hypothesized as being the result of a synergistic or additive effect of the constituents in the extract.

Hu and Kitts (*21*) documented some antioxidant activity of *Echinacea* extracts on human LDL oxidation in vitro, but no attempts were made to correlate the antioxidant activity with the presence in the extracts of specific substances. There are no data available on the specific effects of the individual types of substances present in *Echinacea* on human LDL oxidation. Because oxidative modification of LDL is conducive to atherosclerosis, and thus linked to heart disease, any indications that *Echinacea* substances may protect human LDL from oxidation could provide inspiration for studying the effect of *Echinacea* on other cardioprotective markers and, hence, point at possible new physiologically beneficial effects of *Echinacea*.

The purpose of the present study was therefore to investigate the antioxidant activity, and possible synergistic effects, of extracts, fractions, and pure compounds from *E. purpurea* root in the LDL $CuSO_4$ oxidation assay.

MATERIALS AND METHODS

Plant Material. Four freshly harvested *E. purpurea* roots were supplied by Hans Peter Abrahamsen (Mercurialis, Sorø, Zealand, Denmark). The plants were grown with biodynamic (organic) compost as fertilizer on heavy clay soil at Mercurialis. They were propagated from seeds (Bingenheimer Saatgut AG, Partie 0323702) in a greenhouse for 7 weeks before planting in June 2002. The roots were harvested and washed in cold tap water in March 2003. They were stored dry at a temperature below 5 °C for 4 days during transport to Oslo, Norway, where they were stored in ethanol (96% ethanol, at 5 °C) overnight for preservation. The ethanol was discarded, and the roots were lyophilized until stable weight, pulverized to a fine powder by a mechanical grinder, and stored in closed vessels below −18 °C until extraction.

Extraction. The extraction and fractionation procedure is outlined in **Scheme 1**. All extractions were performed under reflux and with stirring (in a giant rotavapor without suction). The extracts were filtered through Whatman GF/A glass fiber filter.

80% Ethanol Extract. The relatively lipophilic constituents such as phenolics and alkamides were removed from the root material of *E.*

Scheme 1. Flowchart of Extraction and Fractionation Procedure

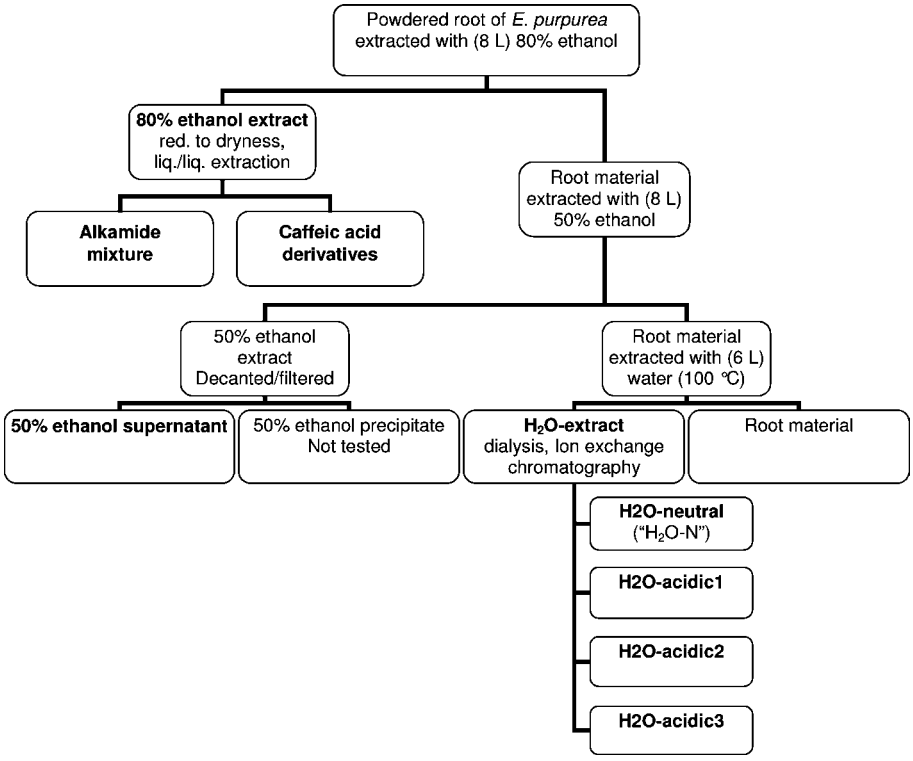

Table 1. Yield (Dry Weight) and Cichoric Acid and Alkamide Content in Extracts and Fractions

	yield		content, mg/g of root[a] (% of dry extract)		
extract/fraction	g[b] (%)	%	cichoric acid	alkamide **2**	alkamide **8/9**
80% ethanol ex	31.4 (34.6)	61.9[c]	13.6 (3.9)	1.7 (0.49)	2.3 (0.66)
50% ethanol supern	11.023 (12.1)	21.7[c]	1.8 (1.47)	nd[d]	nd
50% ethanol prcp	4.3 (4.7)	8.5[c]			
H_2O-ex	4.00 (4.4)	7.9[c]	nd	nd	nd
total extractable	50.723 (55.9)	100			
H_2O-N	0.5375	19.2[e]			
H_2O-acidic 1	0.2842	10.2[e]			
H_2O-acidic 2	1.1175	39.9[e]			
H_2O-acidic 3	0.1654	5.9[e]			

[a] Determined by HPLC. [b] Yield, dry weight g (%) obtained from 90.8 g of dry root material. [c] Percent of extractable. [d] Not detected. [e] Percent of the 2.8 g of H_2O-ex applied in total on the ion exchange-column.

purpurea (90.8 g) by extraction with 2 L of boiling 80% aqueous ethanol four times for 1 h—until only neglible colorization of the last extract. The combined 80% ethanolic extracts were reduced in vacuo to <200 mL, whereby a precipitate formed, which was not possible to separate from the extract by centrifugation/filtration. The concentrated extract (with precipitate) was diluted with water and ethanol (added for preservation of the low molecular compounds) to give 500 mL of a water/ethanol (1:1) solution called "80% ethanol ex". This solution was stored at 5 °C in a brown glass bottle and thoroughly mixed before being used for testing. The concentration, determined to be 62.74 g/L (**Table 1**, 3.137 g of dry weight in 50 mL), was used in calculations of the dilution factor.

50% Ethanol Extract. The residual root material was extracted with 2 L of boiling 50% aqueous ethanol four times for 1 h to remove the less lipophilic compounds and polysaccharides soluble in 50% ethanolic solutions (further compounds expected to be present in common commercial ethanolic tinctures). During storage of the combined 50% ethanol extracts at 5 °C a precipitate formed. The supernatant was separated from the precipitate ("50% ethanol prcp") by decanting/centrifugation and then reduced in vacuo to a volume of <200 mL and diluted with water to a known volume of 250 mL; an aliquot of 50 mL was lyophilized (yield in 50 mL = 2.2046 g ≈ total yield = 11.023 g, **Table 1**). This dry sample, called "50% ethanol supern", was stored in the dark at room temperature and used for testing. The precipitate was not tested in the LDL $CuSO_4$ oxidation assay.

H_2O Extract. Finally, the residual root material was extracted three times with 2 L of boiling water for 1 h, to obtain the ethanol-insoluble compounds such as polysaccharides. The combined water extracts were concentrated in vacuo (at 35−40 °C) and dialyzed at a cutoff of 3500 Da to remove low molecular compounds. After dialysis, the extract was diluted with water to a volume of 500 mL. From this an aliquot of 100 mL was lyophilized (yield in 100 mL = 0.800 g ≈ total yield 4.00 g, **Table 1**); this dry sample called "H_2O-ex" was stored at room temperature in the dark and used for testing. The remaining water extract was divided into aliquots and stored at −18 °C.

Ion-Exchange Chromatography of the H_2O Extract. An aliquot of 100 mL of H_2O-ex filtered through an Acro 50A 5-μm filter (Gelman Sciences) and thereafter through a Millex-HA 0.45-μm filter (Millipore) was applied by pumping to a DEAE-Sepharose fast-flow column (∅ = 5 cm, L = 14.5 cm; Amersham Pharmacia). The column was washed with ∼600 mL of water, resulting in one neutral fraction ("H_2O-N"). Elution of the column with a gradient from 0 to 1 M NaCl in 2 L, at 2 mL/min, resulted in three acidic polysaccharide fractions ("H_2O-acidic 1", "H_2O-acidic 2", "H_2O-acidic 3"**)**. The carbohydrate profile was determined using the phenol−sulfuric acid method (*28*). The absorption at 490 nm was plotted as a function of elution volume (see **Figure 2**), and the fractions were separated according to this. The procedure was repeated another three times (2.8 g applied in total). The elution profile was reproducible, and the relevant fractions were pooled. The acidic fractions were dialyzed (cutoff = 3500 Da) to remove NaCl. All four fractions were reduced in vacuo, lyophilized, weighed, and stored in brown glass containers at room temperature. The yields are given in **Table 1**.

Determination of Monosaccharide Composition by Methanolysis and GC. The polysaccharide samples were subjected to methanolysis with 4 M HCl in anhydrous methanol for 24 h at 80 °C as described by Chambers and Clamp (*29*). Mannitol was added as an internal standard. The trimethylsilyl derivatives were subjected to gas chromatography as described by Samuelsen et al. (*30*).

Quantitative Determination of Total Amounts of Phenolic Compounds. The amount of total phenolics was determined according

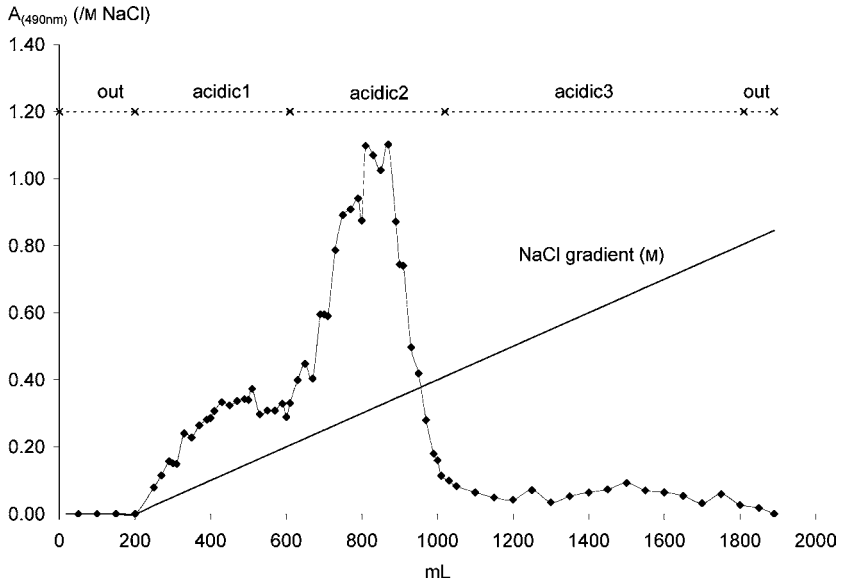

Figure 2. Carbohydrate profile from the ion-exchange chromatography of the H_2O extract from *E. purpurea* root, using the phenol–sulfuric acid method. Absorbance at 490 nm as a function of elution volume, and NaCl concentration (molar) in the eluate as a function of elution volume. The fractions H_2O-acidic 1, H_2O-acidic 2, and H_2O-acidic 3 were collected as illustrated.

to the Folin−Ciocalteu assay (*31*) and modified as described by Rombouts et al. (*32*). The absorbance was measured at 750 nm in a 4049 Novaspec spectrophotometer (LKB Biochrom). The standard curve was plotted using ferulic acid. The total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

References and Pure Compounds. Cichoric acid and a mixture of alkamides was obtained from the 80% ethanol extract by liquid/liquid extraction and pH adjustment as described by Bergeron et al. (*33*) followed by preparative HPLC to obtain pure cichoric acid and derivatives I and II. The alkamide mixture was stored in solution (ethyl acetate) in the dark at 5 °C. Before testing, an aliquot was dried by N_2 flow, and the residue was weighed and redissolved in ethanol/water (1:1). Echinacoside was isolated according to a method developed in the Department of Pharmacognosy, School of Pharmacy, University of Oslo, Norway (to be published). Briefly, 80% ethanol ex of *E. pallida* was taken to dryness and partitioned between water and organic solvents. The butanol fraction was chromatographed over Diaion-HP-20 using a water−methanol gradient. Fractions were combined as indicated by TLC, and the echinacoside fraction was further purified by polyamide column chromatography. Identity and approximate purity (>95%) of isolated echinacoside were determined by NMR spectroscopy. Rosmarinic acid was from Extrasynthèse (Genay, France). Caffeic acid and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO).

HPLC analysis was performed as described by Mølgaard et al. (*34*) (**Figure 3**).

Preparative HPLC was performed on a Dionex system (ASI-100 automated sample injector, P580 pump, Foxy Junior fraction collector, Chromeleon Client v. 6.30 software; Dionex, Sunnyvale, CA) equipped with a Supelco Discovery C_{18} column (25 cm × 21.2 mm, 5 μm) using the following gradient elution: from 0 to 35 min, isocratic B = 20%; from 35 to 50 min, a linear gradient to 80% B; from 50 to 55 min, isocratic B = 80%; from 55 to 56 min, linear gradient to 20% B; and finally equilibration at 20% B for 9 min. Eluent A was acetonitrile/water/trifluoracetic acid (50:949:1), and B was acetonitrile/water (95:5) (acetonitrile was HPLC far-UV quality from Labscan, Dublin, Ireland; water was Milli-Q; trifluoracetic acid was spectranal from Riedel de Häen, Seelze, Germany). With a flow rate of 10 mL/min and an injection volume of 200 μL the cichoric acid and derivatives were collected in the 18−40 min interval. The fractions collected from 29 injections were combined fractionwise, and the eluent was removed in vacuo followed by lyophilization. The purity of the isolated compounds was estimated by analytical HPLC, and their identity was tentatively determined by 1D and 2D NMR.

Preparation of LDL. The LDL was isolated from a pool of plasma samples stemming from blood samples obtained from a large group (>50 persons) of healthy, nonsmoking volunteers, who had not been restricted in their fruit and vegetable consumption prior to blood sampling (Gentofte hospital, Denmark). The LDL (density = 1.019−1.063 g/mL) was isolated from the plasma collected in EDTA (1 mg/mL) by density ultracentrifugation (18 h at 40000 rpm, 4 °C in a Beckman, L8-70M Ultracentrifuge, Beckman, Palo Alto, CA) following the procedure described by Princen et al. (*35*). The LDL protein concentration of the combined purified samples was determined using the Lowry method (*36*), and the sample was deoxygenated by flushing gently with N_2 and stored at 4 °C. To minimize the loss of lipophilic antioxidants, the LDL was not dialyzed prior to oxidation (*37*). A fresh stock solution of 0.2 mg/mL LDL in 0.01 mol/L phosphate-buffered saline (PBS) 0.15 mol/L NaCl, pH 7.4, was prepared every morning and stored at 4 °C under N_2 between assays.

Antioxidant Activity of Samples in Vitro. In quartz cuvettes PBS and LDL stock solution was mixed with 10 μL of diluted sample. The cuvettes were thermostated at 37 °C in a thermostat-controlled automatic sample charger in a UV−vis spectrophotometer (model λ20, Perkin-Elmer, Norwalk, CT), and the oxidation was initiated by adding $CuSO_4$ solution followed by short mixing (giving final concentrations of 0.05 mg of LDL protein/mL and 5 μmol of $CuSO_4$/L). The oxidation was evaluated by monitoring the formation of conjugated diene lipid hydroperoxide, by recording the absorbance at 234 nm every 30 s during 5 h. To obtain dose−response results as presented in **Figure 4** or combination results as presented in **Figures 5** and **6**, the total sample volume was increased to 20 or 30 μL, and the PBS volume was reduced accordingly to give the same final volume. Parallel with all samples a corresponding amount (10, 20, or 30 μL) of water or ethanol/water (1:1) was run as negative control. Gallic acid (in a final concentration of 2 μM) was run at least once a day as a positive control.

The lag time of the tested samples was determined graphically as the *x*-intercept of the tangent to the propagation curve, using Excel (see **Figure 4**). As a measure of the antioxidant activity the net lag phase prolongation (LPP) was calculated as lag $time_{(sample)}$ − lag $time_{(control)}$.

For the investigation of the antioxidant activity of combinations of *Echinacea* constituents, cichoric acid and echinacoside were chosen to represent the caffeic acid derivatives because they are the major caffeic acid derivatives in the three commercial *Echinacea* species. The alkamides were represented by the alkamide mixture from *E. purpurea* root, and the high molecular compounds, polysaccharides and glycoproteins, were represented by the dialyzed lyophilized water extract,

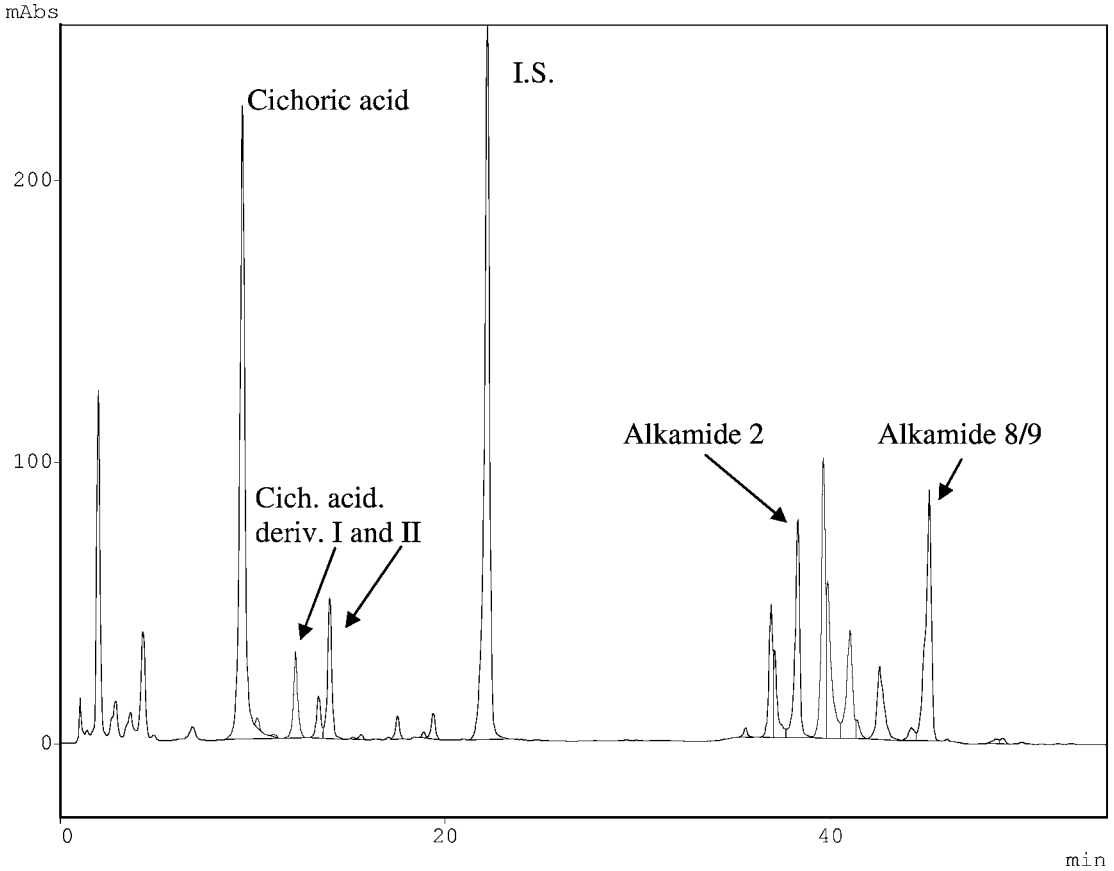

Figure 3. HPLC chromatogram of 80% ethanol ex. HPLC method was as described by Mølgaard et al. (*34*); the detection wavelength was 290 nm for t < 35 min and 260 nm for t > 35 min. I.S. (internal standard) = naringenin. [Alkamide numbers as in Bauer (*9*): alkamide **2**, undeca-2*Z*,4*E*-dien-8,-10-diynoic acid isobutylamide; alkamide **8/9**, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide.]

H_2O-ex, from *E. purpurea* root (see **Scheme 1**). The constituents were tested in concentrations showing an individual LPP of 5−30 min in the earlier investigations (that is, 0.10 μM cichoric acid, 0.10 μM echinacoside, 0.56 mg/L alkamide mixture, and 5.56 mg/L H_2O-ex).

The hypothetical additive LPP of a combination was calculated as the sum of the mean of the LPP of the individual substances in the combination. The LPP achieved by a combination was compared to the hypothetical additive LPP with an unpaired t test using GraphPad Prism (v. 3.0, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Analysis of Materials. The *E. purpurea* root material used in the present study contained cichoric acid (15.4 mg/g of dry root, **Table 1**), alkamide 2 (1.7 mg/g of dry root, **Table 1**), and alkamide **8**/**9** (2.3 mg/g of dry root, **Table 1**). The total amount of alkamides (calculated as alkamide **2**) in the 80% ethanol extract was estimated to be 11 mg/g of dry root material. The levels of alkamides and cichoric acid found in the root material used in this study agree well with previously published data for Danish-grown *E. purpurea* (*34*). The HPLC fingerprint of the alkamide mixture isolated from the 80% ethanol extract was identical with the alkamide part of the HPLC chromatogram of the total 80% ethanol extract (t_r > 35 min in **Figure 3**); hence, the obtained alkamide mixture was representative of the alkamides in the 80% ethanol extract of *E. purpurea* root. The presence of trace amounts of caffeic acid derivatives in the alkamide mixture cannot be excluded, because small peaks with t_r < 25 min were observed (results not shown) but were not quantifiable.

The yield and purity of the cichoric acid derivatives isolated from the 80% ethanol extract by liquid/liquid extraction and preparative HPLC that were tested in the LDL $CuSO_4$ oxidation assay were (yield, purity determined by analytical HPLC) cichoric acid (30 mg, 99.5%), derivative I (6.5 mg, 92%), and derivative II (10 mg, 84%). The cichoric acid derivatives I and II (see peak assignment in **Figure 3)** were tentatively determined by comparison of NMR spectra with the literature (*38*−*41*) to be I, 2-caffeoyl-3-*p*-coumaroyl tartaric acid, and II, 2-caffeoyl-3-feruloyl tartaric acid.

The carbohydrate percentage of dry weight increased as expected with increasing water content in the extractant (see **Table 2**): The 80% ethanol extract contained 26.1% of carbohydrate, mainly glucose, whereas the 50% ethanol extract contained 44.7% of carbohydrate dominated by galacturonic acid, arabinose, glucose, and galactose, and the water extract (H_2O-ex) contained 79.8% of carbohydrate dominated by arabinose and galacturonic acid. Following ion exchange of the H_2O-ex the amount and profile of the carbohydrates were unevenly distributed among the four fractions H_2O-N containing 39.2% of carbohydrates, H_2O-acidic 1 containing ∼100%, H_2O-acidic 2 containing 93.2%, and H_2O-acidic 3 containing 49.6% of carbohydrates (see **Table 2** for profiles). Previously, a raw polysaccharide fraction has been isolated from *E. purpurea* root (*42*); detailed characterization of this fraction has not been published, but it is believed (*9*) to be similar in composition to the polysaccharides from the arial parts. Two polysaccharides have been isolated from the aqueous extract of the arial parts: PSI (a 4-*O*-methyl-glucoronoarabinoxylan, M_w ∼ 35 000 Da) and PSII (an acidic arbinorhamnogalactan, M_w ∼ 450 000 Da) (*9*). From the roots of *E. purpurea* and *E. angustifolia* three

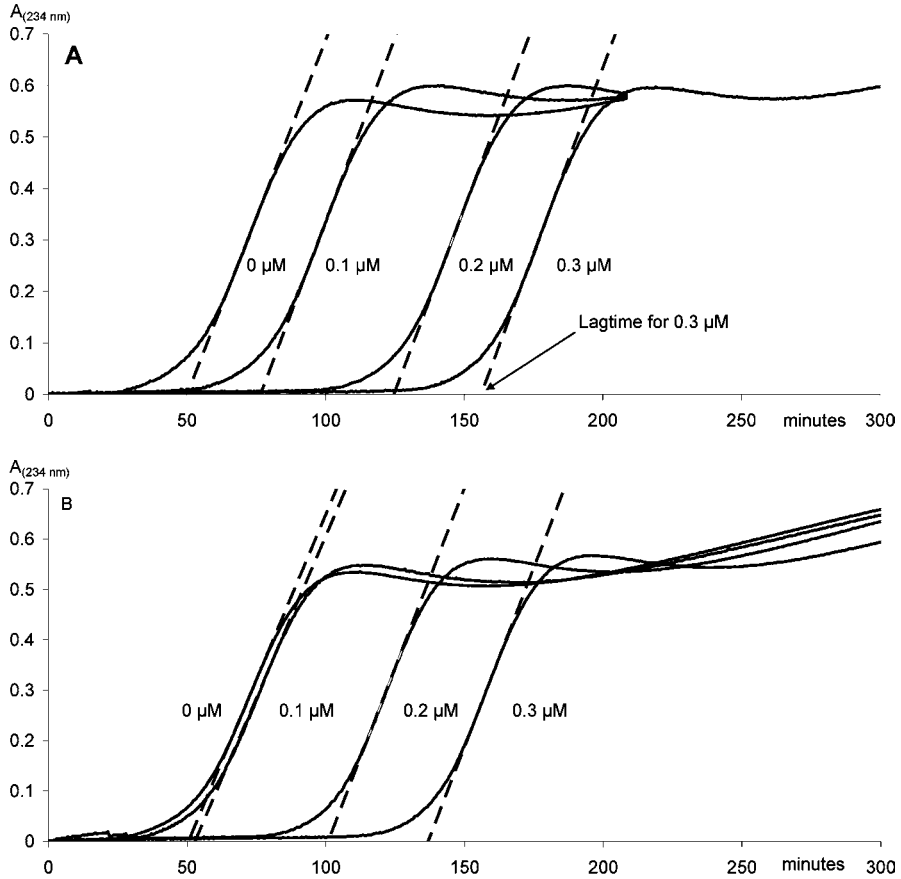

Figure 4. Inhibition of Cu(II)-catalyzed LDL oxidation by (**A**) cichoric acid and (**B**) echinacoside at three levels (A_{234nm} versus time) (representative examples) compared with zero antioxidant addition. The formation of conjugated dienes (A_{234nm}) during oxidation results in an increase in the absorbance. The lag time was determined as the *x*-intercept of the tangent to the propagation curve. The lag-phase prolongation was determined as lag time$_{(sample)}$ − lag time$_{(control\ without\ antioxidant)}$. Data such as these were used to calculate the dose–response relationships in **Table 5**.

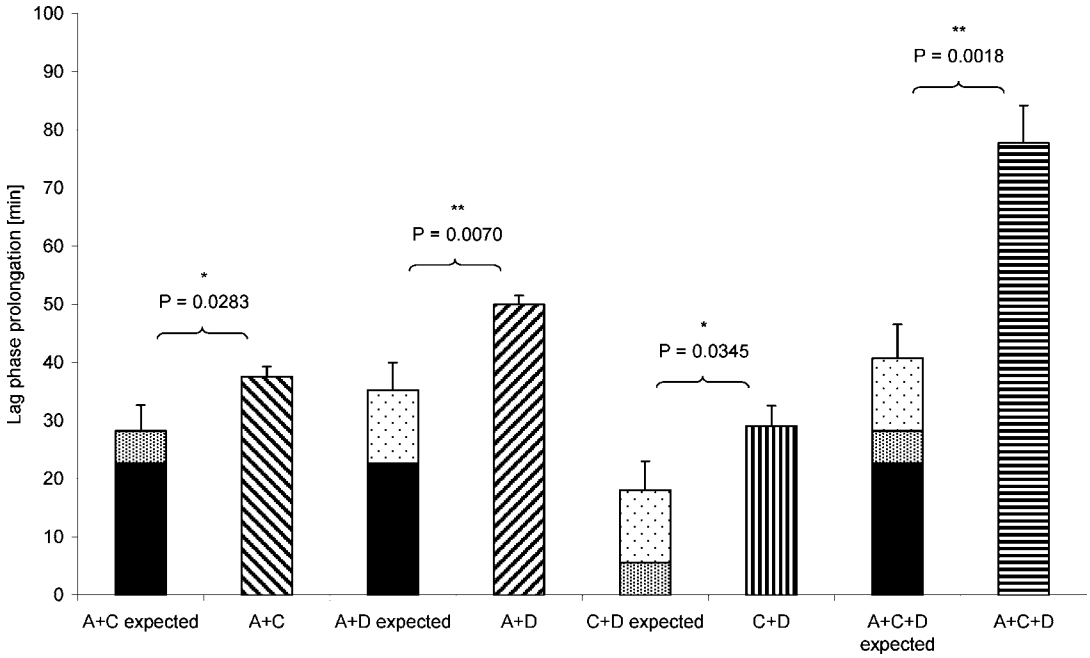

Figure 5. Antioxidant activity of combinations of (A) cichoric acid (0.10 μM), (C) alkamides (0.56 mg/L), and (D) H_2O extract containing polysaccharides (5.56 mg/L) measured as lag phase prolongation of LDL samples during copper-induced oxidation. All combinations were significantly more active than expected from addition of the individual antioxidant activities (unpaired *t* test using GraphPad Prism; *, $P < 0.05$; **, $P < 0.01$).

glycoproteins (M_w 17 000, 21 000, and 30 000 Da) were isolated; the dominant sugars in these were arabinose, galactose, and glucosamines (*9*). Although our fractions seem to contain the relevant monosaccharides to be of similarity to the previously published polysaccharides from *E. purpurea* herba or the glycoproteins from *E. purpurea* and *E. angustifolia* root, further characterizations of our fractions are needed to confirm or reject this.

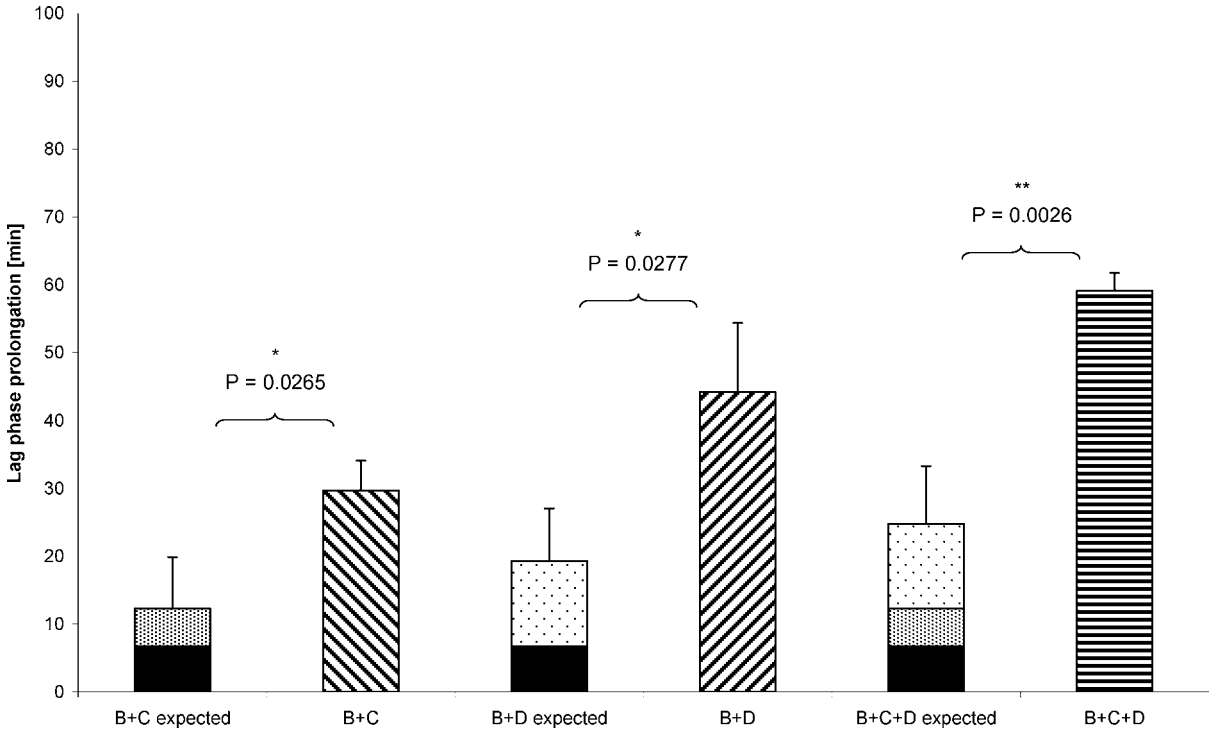

Figure 6. Antioxidant activity of combinations of (B) echinacoside (0.10 μM), (C) alkamides (0.56 mg/L), and (D) H_2O extract containing polysaccharides (5.56 mg/L) measured as LPP of LDL samples during copper-induced oxidation. All combinations were significantly more active than expected from addition of the individual antioxidant activities (unpaired *t* test using GraphPad Prism; *, $P < 0.05$; **, $P < 0.01$).

Table 2. Monosaccharide Composition (Percent of Total Carbohydrate Content) and Total Phenol (Percent) of Extracts and Fractions from *E. purpurea* Root

	80% ethanol ex	50% ethanol supern	50% ethanol prcp	H_2O-ex	H_2O-N	H_2O-acidic 1	H_2O-acidic 2	H_2O-acidic 3
Ara	0.0	25.0	7.3	49.0	80.6	38.9[a]	34.7	50.8
Rha	0.0	4.4	1.3	5.9	0.0	12.0	7.0	9.0
Xyl	0.0	1.7	0.0	1.4	0.0	6.0	0.6	2.0
Man	0.7	2.2	1.9	0.1	0.0	0.2	0.0	0.0
Gal	10.5	9.0	5.3	6.2	10.2	15.3	7.3	15.6
Glc	85.7	13.6	67.9	1.9	0.0	1.0	0.0	1.4
GalA	3.1	44.1	17.2	35.6	6.1	26.6	49.8	21.2
GlcA	0.0	0.0	0.0	0.0	3.1	0.0	0.6	0.0
total amount of carbohydrate in sample (μg)	260.7	446.9	53.4	957.5	431.5	1476.5	931.5	495.8
carbohydrate (% of dry wt)	26.1	44.7	5.3	79.8	39.2	~100	93.2	49.6
total phenolics[b] (% of dry wt)				2.6	trace	trace	trace	3.0

[a] The value is beyond the linear range of the standard curve. [b] Determined as ferulic acid equivalents (FA/sample) × 100%.

Table 3. Antioxidant Activity of Caffeic Acid Derivatives from *Echinacea*, LPP at Three Concentrations[a]

	0.01 μM	0.10 μM	1.00 μM
caffeic acid	1.7 ± 1.0	4.9 ± 1.7	227.0 ± 18.6
cichoric acid	4.9 ± 1.7	28.8 ± 5.7	>300
derivative I	−1.7 ± 3.1	2.3 ± 2.1	101.0 ± 2.6
derivative II	3.2 ± 1.1	10.9 ± 2.2	223.2 ± 22.4
echinacoside	−1.5 ± 0.3	11.4 ± 6.9	241.6 ± 20.0
	0.25 μM	0.50 μM	1.00 μM
rosmarinic acid	42.5 ± 5.2	107.7	210.3 ± 34.2

[a] Caffeic acid and rosmarinic acid were included for comparison. The positive control, 2 μM gallic acid, gave a LPP of 74.6 ± 15 min; 1 μM caffeic acid equals 0.18 mg/L, 1 μM rosmarinic acid equals 0.36 mg/L, 1 μM cichoric acid equals 0.47 mg/L, and 1 μM echinacoside equals 0.79 mg/L. Dilutions of derivatives I and II were made using the M_w for cichoric acid.

The light brown color of the H_2O-acidic 3 fraction led us to investigate the total phenolic content of the H_2O-ex and its fractions: the H_2O-ex and the H_2O-acidic 3 contained 2.6 and 3.0% of phenolics, respectively, whereas the other H_2O fractions contained only trace levels of phenolics (see **Table 2**).

Antioxidant Activity of Isolated Caffeic Acid Derivatives. An increase in the concentration of antioxidant present in the cuvette during $CuSO_4$-induced oxidation of LDL will result in a longer lag time before oxidation of the LDL. All of the tested compounds exerted a dose−response effect in the LDL assay (as exemplified for cichoric acid and echinacoside in **Figure 4).**

Cichoric acid was by far the most potent antioxidant, and its antioxidant activity surpassed that of the others at all of the tested dose levels (1, 0.1, and 0.01 μM addition levels, see **Table 3**). Hence, at 1 μM cichoric acid totally inhibited the Cu^{2+}-induced LDL oxidation during the measured time period of 300 min. Rosmarinic acid, caffeic acid, derivative II, and echinacoside showed intermediate activity, whereas derivative I was the least potent antioxidant (**Table 3**). Hence, the order of antioxidant activity of the tested phenylpropanoids in the LDL oxidation assay was cichoric acid > echinacoside ≥ derivative II ≥ caffeic acid ≥ rosmarinic acid > derivative I. The change in order of activity between cichoric acid and echinacoside and between rosmarinic acid and echinacoside, when compared to the previously reported order of antioxidant activity (echinacoside > cichoric acid > cynarin > chlorogenic acid > caffeic acid > caftaric acid) in the DPPH$^\bullet$ assay (*19*) and (rosmarinic acid > ... > echinacoside) in the Briggs−Rauscher method (*22*), is most likely due to differences between the assays used. This difference may result in different antioxidant mechanisms being dominant, for example, the DPPH$^\bullet$ assay measures radical

Table 4. Antioxidant Activity of Extracts and Fractions, LPP at Three Concentrations

	0.56 mg/L	5.56 mg/L	22.22 mg/L
80% ethanol ex	6.4 ± 1.5	247.3 ± 27.8	
alkamide mixture	6.4 ± 2.4	222.5 ± 6.6	>300
50% ethanol supern	1.6 ± 0.9	23.2 ± 2.2	154.4 ± 13.9
H_2O-ex	0.2 ± 0.9	11.8 ± 2.3	72.4 ± 6.9
H_2O-N		1.0 ± 1.4[a]	2.9 ± 1.1[b]
H_2O-acidic 1		0.2 ± 2.7	0.8 ± 0.5
H_2O-acidic 2		−1.8 ± 0.5	0.7 ± 1.3
H_2O-acidic 3	1.1 ± 0.1	10.4 ± 2.4	117.4 ± 14.6

[a] Tested at 7.4 mg/L. [b] Tested at 29.6 mg/L.

scavenging in only a uniphasic, aqueous system, whereas the inhibition of Cu^{2+}-catalyzed LDL oxidation by caffeic acid derivatives represents both their metal chelation and radical scavenging abilities (*25*). However, because the tested concentrations of phenylpropanoids in our study did not exceed the added concentration of copper (5 μM), the observed antioxidant activity could not be solely due to metal chelation as suggested by Andreasen et al (*23*). The differences in the antioxidant activities on LDL oxidation could also be ascribed to differences in solubilities and partitioning behavior between the aqueous and lipid phases in the LDL antioxidant test system: Hence, the physicochemical properties of the different types of antioxidant phytochemicals may have influenced their efficacy as previously descibed for antioxidant activity in oils versus in emulsion systems (*43*). In addition, as previously proposed, structural features conferring differences in protein binding to tryptophan residues in the LDL−apolipoprotein B may also explain differences in antioxidant activity among phenolics in inhibiting oxidation of human LDL (*44*).

Looking at the structure−activity relationship between the tested phenylpropanoids, the importance of the *o*-dihydroxy functionality to achieve good antioxidant activity is confirmed by the order of activity of the cichoric acid derivatives: cichoric acid (2,3-dicaffeoyltartaric acid containing two *o*-dihydroxy functionalities) > derivative II (2-caffeoyl-3-feruloyl tartaric acid containing one *o*-dihydroxy and one *o*-hydroxymethoxy functionality) > derivative I (2-caffeoyl-3-*p*-coumaroyltartaric acid containing one *o*-dihydroxy and a single hydroxy functionality). However, other structural features besides the *o*-dihydroxy must play a part because caffeic acid (with one *o*-dihydroxy), rosmarinic acid, and echinacoside (both containing two *o*-dihydroxy groups) all show antioxidant activity similar to that of derivative II.

All of the tested *Echinacea* phenylpropanoids showed higher antioxidant activity (at 1 μM) than the positive control gallic acid (at 2 μM), which corresponded well with the findings in the literature (*25*) that cinnamic acid derivatives tended to be better antioxidants than their benzoic acid counterparts. At the concentrations tested by Andreasen et al. (*23*) (5−40 μM) caffeic acid and gallic acid both inhibited the Cu^{2+}-induced oxidation of LDL for the entire measured time period, and hence a ranking of the two from these data was not possible.

Antioxidant Activity of Extracts and Fractions from *E. purpurea* Root. Among the extracts and fractions from *E. purpurea* root the 80% ethanol extract and the alkamide mixture showed the highest antioxidant activity (**Table 4**). For the 80% ethanol extract the lag time at 5.56 mg/L was close to the limit of 300 min, and hence no measurement was made at 22.22 mg/L. Instead, the 80% ethanol extract was tested at 0.056 mg/L, but no lag time prolongation was seen at this low concentration (results not shown). The high antioxidant acivity of the 80% ethanol extract from *E. purpurea* root was predictable because it was expected to contain the phenylpropanoids, well-known as antioxidants. Knowing from HPLC analysis (**Table 1**) that 3.9% of the extract's dry weight is cichoric acid and using the dose−response relationship for cichoric acid in **Table 5**, a theoretical LPP of 168 min can be calculated for the 80% ethanol extract at 5.56 mg/L; the experimental value is 247.3 ± 27.8 min. Hence, the majority of the antioxidant activity of the 80% ethanol extract can be explained by the cichoric acid content, and the rest of the activity can probably be explained by the presence of other phenylpropanoids and/or the alkamides (see **Figure 3**).

The alkamide mixture was effectively protecting the LDL against oxidation for more than the observed 300 min at the highest tested concentration (22.22 mg/L ~ 97 μM). The tested concentrations of the alkamide mixture were estimated to be 2.4, 24, and 97 μM, respectively, using M_w (alkamide 2) ≈ 230 g/mol. The alkamide mixture at 24 μM (5.56 mg/L) resulted in a LPP of ~225 min (**Table 4**), which was similar to the LPP obtained by 1 μM rosmarinic acid (**Table 3**). Hence, the antioxidant activity of the alkamide mixture was weaker than that of the tested caffeic acid derivatives by a factor of 24. No antioxidant activity of the alkamide mixture from *E. purpurea* root was expected because no former reports of good antioxidant activity of alkamides had been published. On the contrary, Thulin and Thygesen (*26*, *27*) detected no antioxidative effect of a similar alkamide mixture from a commercial extract of *E. purpurea* root. Hu and Kitts found no radical scavenging activity in the $CHCl_3$ extracts of *Echinacea* species, and hence they suggest that the alkamides "are not likely to be the principal components contributing to free radical scavenging activity" (*21*). It must be admitted that trace amounts of caffeic acid derivatives present in the alkamide mixture may contribute to some extent to the unprecedented antioxidant effect of the alkamide mixture seen in the present study. However, the lipophilic nature of the alkamides may also suggest that the effect of the alkamide mixture could be due to their ability to mix with the LDL and, perhaps, indirectly modify its ability to be oxidated.

Table 5. Linear Relationship, $Y = \alpha X - \beta$, between Lag Time Prolongation (LPP) and Antioxidant Concentration, where Y Is the Resulting LPP, the Slope Is the Lag Time Increase per Micromolar Antioxidant, X Is the Concentration of Antioxidant (Micromolar), and β Is the Intercept on the Y Axis[a]

constituent	t_{LPP} [min] =	r, P
cichoric acid (A)	(367.3 ± 21.3 [min/μM]) × (concn [μM] − 3.9 ± 3.4 [min])	0.974, <0.0001
echinacoside (B)	(249.9 ± 8.4 [min/μM]) × (concn [μM] − 4.9 ± 3.5 [min])	0.990, <0.0001
alkamide mixture (C)	(42.1 ± 1.8 [min/(mg/L)]) × (concn [mg/L] − 19.0 ± 4.3[min])	0.986, <0.0001
H_2O extract (D)	(3.9 ± 0.3 [min/(mg/L)]) × (concn [mg/L] − 2.9 ± 3.5 [min])	0.948, <0.0001

[a] Letters in parentheses refer to **Figures 5** and **6**. Regression was based on data for cichoric acid at 0, 0.01, 0.10, 0.20, and 0.30 μM; echinacoside at 0, 0.01, 0.10, 0.20, 0.30, and 1.0 μM; the alkamide mixture at 0, 0.56, 1.11, 1.67, and 5.56 mg/L; and the H_2O extract at 0, 0.56, 5.56, 11.11, 16.67, and 22.22 mg/L.

The 50% ethanol supernatant, the H_2O extract, and the H_2O-acidic 3 fraction showed a dose-dependent low to medium antioxidant activity. The H_2O-acidic 1, H_2O-acidic 2, and H_2O-N fractions were tested only at the two highest concentrations because they showed very low or no LPP. The antioxidant activity of the 50% ethanol supernatant was low compared to the expected LPP; for example, at 5.56 mg/L the experimental value was 23.2 ± 2.2 min, whereas the expected value was 63 min (calculated using a cichoric acid content of 1.47%, **Table 1**, and the dose response relationship for cichoric acid in **Table 5**). This could be due to antagonistic effects of other constituents in the 50% ethanol supernatant or to decomposition of cichoric acid in the dried 50% ethanol supernatant during storage.

The water extract and fractions contained acidic polysaccharides, and it is well-known that acidic polysaccharides with a high content of uronic acid can be responsible for some heavy metal chelation (*45*). The antioxidant activity exhibited by the H_2O-ex and the H_2O-acidic 3 is not likely to be due to chelation of Cu^{2+} by these acidic polysaccharides, because no significant activity was found in the other acidic fractions from the water extract (H_2O-acidic 1 and H_2O-acidic 2). The difference in antioxidant activity of the H_2O extract and fractions could well be due to the presence of 2.6 and 3% phenolics in the H_2O-ex and H_2O-acidic 3, respectively (**Table 2**). The antioxidant activity of the phenolics associated with the polysaccharides in these fractions was not comparable to that of cichoric acid, because the expected LPP (calculated as 5.56 mg/L containing 3% cichoric acid and using the dose response relation for cichoric acid in **Table 5**) was 129 min compared to the experimental results of 11.8 ± 2.3 min for H_2O-ex and 10.4 ± 2.4 min for H_2O-acidic 3 (**Table 4**).

The order of activity of the tested extracts and fractions (at a concentration of 5.56 mg of dry weigth/L) was 80% ethanol ex > alkamide mixture ≫ 50% ethanol supern > H_2O-ex > H_2O-acidic 3 ≫ H_2O-N ≈ H_2O-acidic 1 ≈ H_2O-acidic 2. The same pattern was seen at 22.22 mg/L, although at this concentration H_2O-acidic 3 exhibited a higher activity than H_2O-ex.

Antioxidant Activity of Combinations of Cichoric Acid or Echinacoside with Alkamides and/or Polysaccharide Fractions. When tested separately, cichoric acid, echinacoside, the alkamide mixture, and the H_2O extract showed a dose-dependent LPP as described by the respective functions in **Table 5**. In various combinations they all showed a significantly higher LPP than expected from addition of the individual values (**Table 6** and **Figures 5** and **6**). The combination of cichoric acid and alkamides exhibited a 33% higher activity relative to the expected additive LPP; cichoric acid and the H_2O extract in combination resulted in a 42% relative increase, whereas all three (cichoric acid, alkamides, and H_2O-ex) combined resulted in a relative increase of 91% (**Figure 5**). The combination of alkamides and H_2O-ex resulted in a 61% increase (**Figure 5**). Echinacoside and alkamides in combination resulted in a 142% increase as compared to the hypothetical additive effect, and echinacoside and H_2O-ex resulted in a 130% increase, whereas all three (echinacoside, alkamides, and H_2O-ex) resulted in a 138% increase (**Figure 6**). Hence, all other things being equal, including endogenous α-tocopherol levels in the LDL, we conclude that the constituents of the *Echinacea* preparations exhibit significant synergistic antioxidant effects in the LDL Cu^{2+} oxidation assay and, in turn, that the antioxidant activity of complex mixtures such as *Echinacea* preparations are caused by a synergistic effect of the constituents in the *Echinacea* extract. The presence of phenolics in the H_2O-ex, and the possible trace amounts of phenolics in the alkamide mixture, may suggest that part of the synergistic antioxidative activity might also be a result of positive synergistic actions among phenolics in the extracts. However, due to the extraction procedure, the phenolics found in the H_2O-ex must be either very polar (they are present in the H_2O-ex, not in the ethanolic extracts) and very acidic, because they elute with the latest eluting fraction on ion-exchange (H_2O-acidic 3), or they must be associated with the polysaccharides. The alkamide mixture contained only unquantifiable trace amounts of phenolics, and hence their contribution to the activity of the alkamide mixture is expected to be relatively small, but perhaps enhanced by the lipophilic nature of the alkamides. Hence, the observed synergistic effects between the pure cichoric acid (or echinacoside) and the H_2O-ex and/or the alkamide mixture are expected to be caused by the constituents present in the extracts and fractions. Further studies with a different study design and analytical setup are required to permit firm conclusions to be drawn regarding the mechanism behind the synergistic effect between the purified chichoric acid and echinacoside and the extracts and fractions.

Table 6. Antioxidant Activity of Individual Constituents and Combinations of Constituents from *Echinacea*.

constituent/ combination[a]		LPP[b] (min)
A	cichoric acid (0.1 μM)	22.7 ± 3.0[c]
B	echinacoside (0.1 μM)	6.8 ± 6.9[c]
C	alkamide mixture (0.56 mg/L ~ 2.4 μM)	5.5 ± 3.3
D	H_2O-ex (5.56 mg/L)	12.5 ± 3.7
A + C	cichoric acid and alkamide mixture	37.5 ± 1.7
A + D	cichoric acid and H_2O-ex	49.9 ± 1.5
A + C + D	cichoric acid, alkamide mixture, and H_2O-ex	77.7 ± 6.5
B + C	echinacoside and alkamide mixture	29.7 ± 4.4
B + D	echinacoside and H_2O-ex	44.2 ± 10.1
B + C + D	echinacoside, alkamide mixture, and H_2O-ex	59.1 ± 2.6
C + D	alkamide mixture and H_2O-ex	29.1 ± 3.5

[a] Capital letters refer to **Figures 5** and **6**. [b] Mean of three determinations ± standard deviation. [c] The difference in the results for echinacoside and cichoric acid at 0.1 μM when compared to the results in **Table 3** is expected to be due to different LDL pools used.

The antioxidant activity of various extracts of *Echinacea* species has previously been tested (*19*−*21*, *27*), but to our knowledge this is the first time that (re)combinations of three groups of *Echinacea* constituents have been tested and the first time that synergistic effects between caffeic acid derivatives, alkamides, and polysaccharide fractions have been demonstrated. The amount of literature hypothesizing synergistic effects of the constituents in extracts is immense, but very few results have in fact been published on the subject. Recently, Milde et al. (*46*) found a synergistic effect of rutin combined with ascorbic acid or γ-terpinene on LPP in Cu^{2+}-mediated LDL oxidation. Previously, an antagonistic effect of caffeic acid and catechin or cyanidin has been reported (*44*). Synergistic antioxidant effects of ferulic acid and ascorbic acid and antagonistic interactions of ferulic acid with α-tocopherol or β-carotene in rat liver microsomal membranes have recently been reported (*47*).

The concentrations tested in combination in vitro in our investigation were chosen, for practical reasons, to give a LPP between 5 and 30 min. The relationship between cichoric acid (0.1 μM = 0.474 mg/L), the alkamide mixture (0.56 mg/L), and the water-extractable polysaccharides/glycoproteins (5.56

mg/L) tested was 0.85:1:10. This appeared to be in the same order of magnitude as the relationship found in the root material (1.54, 1.1, and 4.4%, respectively). The tested concentrations were on average 10 000 times more dilute than the concentrations found in the root material. Practically nothing is known about absorption and distribution in the human body of substances after intake of *Echinacea* extracts. Matthias et al. recently found high permeability in a Caco-2 cell model of intestinal uptake for alkamides and cinnamic acid, but low permeability for the more complex caffeic acid derivatives such as cichoric acid and echinacoside. The polysaccharides were not investigated (*48*). It is very likely that the phenylpropanoid esters (such as cichoric acid and echinacoside) will be chemically hydrolyzed by gastric acid or by esterases yielding the more easily absorbed phenylpropanoids. Cinnamic acid derivatives are ionized at physiological pH (pH 7.4) and thus are highly hydrophilic (Log $D_{7.4} < -1$), decreasing the likely volume of distribution to the plasma, that is, in an adult human, 5 L. The in vitro data obtained in this study cannot be immediately transformed to probable in vivo results. However, if just hypothesizing, it is very interesting to note that using a hypothetical bioavailability of 100% and the above estimated distribution volume of 5 L, the hypothetical concentration of cichoric acid in an adult human being would be 2.4 mg of cichoric acid/L, when the recommended daily dose of 3 mL of an extract is consumed, which contains, for example, 4 mg of cichoric acid/mL [concentration of cichoric acid found in a commercial extract by Mølgaard et al. (*34*)]. Hence, the concentrations tested in the present study were of the same order of magnitude as this very rough estimate of a hypothetical in vivo concentration. Before direct conclusions about in vivo activity can be made, further studies investigating the uptake, distribution, and metabolism of the active constituents from *Echinacea* are needed. However, because this is the first time that actual synergistic effects of the three groups of *Echinacea* constituents have been documented, we find the results to be very promising.

ABBREVIATIONS USED

ABTS$^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; DPPH$^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl radical; LDL, low-density lipoprotein; LPP, net lag phase prolongation.

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