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Antioxidant activity of cichoric acid and alkamides from *Echinacea purpurea*, alone and in combination

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Abstract

The antioxidant activity of extracts of the stems, leaves, and roots of *Echinacea purpurea* was compared with the antioxidant activity of purified cichoric acid and alkamides, both constituents of *Echinacea purpurea*. The antioxidant activity was determined using different methods: effect on oxygen consumption rate of a peroxidating lipid emulsion, and scavenging of radicals, i.e. 2,2-diphenyl-1-picryl-hydrazyl (DPPH), measured by two different techniques. The efficacy of the extracts in the reaction with DPPH correlated well with the amount of cichoric acid present in the various extracts. The alkamides alone showed no antioxidant activity in any of the tests. Alkamides present in the extract increased, however, the antioxidative effect of cichoric acid in the peroxidating lipid emulsion. The activity was further compared with that of rosmarinic acid, a well-characterised antioxidant, and the extracts as well as cichoric acid were found to be efficient scavengers of radicals with an activity comparable to that of rosmarinic acid. Cichoric acid was found to have a stoichiometric factor of 4.0 in scavenging DPPH and to react in a second-order reaction with DPPH with a rate constant of 40 l/mol/s at 25 °C in methanol.

Keywords: *Echinacea purpurea*; Antioxidant activity; Cichoric acid; Alkamides

1. Introduction

Among the various *Echinacea* species, *E. purpurea* is the most common in herbal medicine in Europe. The popularity as an ingredient in functional foods, supplements and certain candies seem to be increasing alongside the appearance of clinical evidence for efficiency against common colds (Brinkeborn, Shah, Geissbühler, & Degenring, 1999; Goel et al., 2004). The activity seems mainly directed towards the non-specific cellular immune system (Bauer & Wagner, 1990), and the effect of *Echinacea* has been verified in several clinical studies. The immunostimulating effect is caused by four reactions: activating phagocytoses, stimulating the fibroblasts, increasing respiratory activity, and increasing mobility of leucocytes (WHO, 1999). However, it has not yet been established in what way extracts of *Echinacea* more specifically affect the various systems of the body. Pharmacology of herbal medicine is rather complex as herbal preparations contain a whole array of chemical compounds working together. Most often there is only a rather incomplete knowledge of their bioavailability, and besides, some of the active components may have remained undetected, or the activity even ascribed to the wrong constituent(s) (Jensen, Hansen, & Nielsen, 2001). All together this makes it often hard to explain the molecular mechanism behind any observed effect. In addition some of the active compounds may create a synergistic effect in the body, which further complicates the understanding of the mechanism (Hobbs, 1989).

In order to determine what makes *Echinacea purpurea* so widely used, several studies have been carried out trying to identify the active compounds and their function(s). So far cichoric acid (di-caffeoyl tartaric acid) (Fig. 1), alkamides

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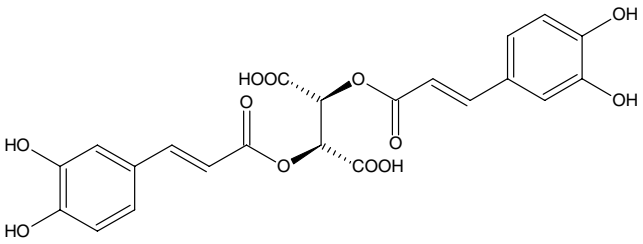
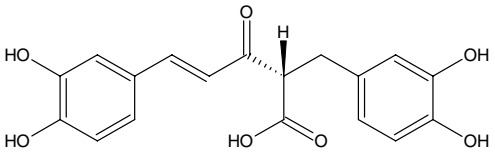
(alkyl amides) (Fig. 1), polysaccharides and glycoproteins are considered as the most important compounds (Bauer, 1999). Extracts of *E. purpurea* have been tested for pharmacological activity in several in vivo and in vitro studies. Most studies are based on the effect of juices from the aerial part of *Echinacea purpurea* on the immune-system (Bauer & Tittel, 1996). However, there still seems to remain much to be learned about the specific mechanisms of effects of *Echinacea purpurea* (Hobbs, 1989; Melchart, Linde, Fischer, & Kaesmayr, 2002).

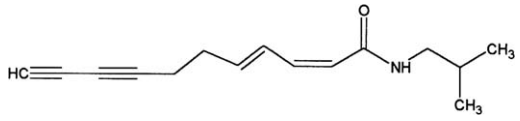
Antioxidants affect the human metabolism in general and have, based on more or less well-documented investigations, been suggested for the treatment of various types of illnesses (Gate, Paul, Ngyen, & Tew, 1999; Baurenová & Bezek, 1999). The antioxidant activity of *E. purpurea* extracts has been investigated (Pietta, Simonetti, & Mauri, 1998), and it was found that the plant extracts were rather inefficient. From a later study, including root extracts from several *Echinacea* species, it was concluded that roots of *E. purpurea* do possess an antioxidative activity, although less than roots of *E. pallida* (Hu & Kitts, 2000). Most recently, Pellati, Benvenuti, Magro, Melegari, and Soragni (2004) have shown that all investigated *Echinacea* species did possess radical scavenging activity, *E. purpurea* being the most efficient. The antioxidant activity could be ascribed to the phenolic content of the roots, and cichoric acid present in *E. purpurea* was almost as efficient as echinacoside in *E. pallida*.



Cichoric acid



Rosmarinic acid



Alkamide 2

Fig. 1. Structure of cichoric acid, rosmarinic acid, and alkamide 2, i.e. undeca-2Z,4E-dien-8,10-diynisobutylamid.

According to Rice-Evans, Miller, and Pagana (1996) phenolic substances like caffeic and chlorogenic acid belong to the most efficient antioxidants from natural sources. Additionally, polyphenols are better antioxidants than monophenolics (See, Broumand, Sahl, & Tilles, 1997; Steinmüller et al., 1993). Substitution of the aromatic ring in ortho- or para-position will enhance the antioxidant efficacy because of the possible resonance structures leading to increased stability of the antioxidant radical formed upon scavenging of other radicals. Caffeic acid has one ortho-dihydroxy phenyl group only, while cichoric acid and rosmarinic are composed of two molecules of caffeic acid (Fig. 1).

In order to obtain more detailed information of antioxidative activity of *E. purpurea*, extracts of various parts of the plant were investigated and compared with isolated cichoric acid and alkamides from *E. purpurea*. Cichoric acid is present only in Asteraceae. Alkamides are found in three families: Piperaceae, Rutaceae, and Asteraceae (Bohlman, Burkhardt, & Zdero, 1973), of which the latter is by far the most important. The present study is a step forward compared to previous studies as we have investigated cichoric acid and alkamides separately as well as in combination. Cichoric acid is in particular interesting in this perspective as already shown by Pellati et al. (2004). As a plant phenolic, it belongs to the largest group of known natural antioxidants (Madsen, Bertelsen, & Skibsted, 1997). It should, however, be noted that only a minor part of the chemical compounds in spices showing antioxidative activity has so far been isolated and identified. For rosemary (*Rosmarinus officinalis* L.), several of the phenolic diterpenes have been shown to possess antioxidative properties, together with rosmarinic acid (Fig. 1). Like cichoric acid, rosmarinic acid is a caffeic acid derivative (Fig. 1), and rosmarinic acid is used commercially in form of partly purified rosemary extracts which have both antioxidative and antimicrobial activity (Madsen et al., 1997). The well established use of rosmarinic acid preparations makes purified rosmarinic acid an obvious reference for the activity of cichoric acid, which also may have potential use in foods. The investigation follows the strategy recently outlined for antioxidant evaluation, as the concentration of potential antioxidants were determined as step one, followed by a static and dynamic determination of radical scavenging as the second step, and by effect in a lipid emulsion model system as the third step (Becker, Nissen, & Skibsted, 2004). An evaluation of the in vivo effect (step IV b) is currently being conducted.

2. Materials and methods

2.1. General

The plant material derived from two cultivations of *E. purpurea* cv. Verbesserte Leuchtstern. One batch was grown from seeds (Jelito®) and planted out on clay soil at Pajbjerg, Denmark (55°N). The other batch was grown

on moist meadow soil in Taastrup, Denmark (55°N), using root cuttings of the first batch. Tops and roots were harvested in 1998, and kept cool in darkness until use. The extracts were quantified by HPLC (Table 1), and tested directly in the antioxidant assay. All samples had to be made from new for each assay. These plant extracts were compared with composed test solutions made to simulate the natural concentrations of cichoric acid and alkamides, as shown in Table 2. Cichoric acid was tested in two concentrations, C1 and C2, the alkamides in three, A1–A3, and cichoric acid in low concentration was tested with all three alkamide concentrations C1A1, C1A2, and C1A3. The alkamide levels in the test samples were decided on according to results with Danish grown plants (cf. Table 1).

2.2. Reagents

Metmyoglobin (MMb, type III, from horse heart), methyl linoleic acid and Tween-20 was supplied by Sigma (St. Louis, MO, USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) was from Aldrich (Vallensbæk Strand, Denmark), and rosmarinic acid ($C_{18}H_{16}O_8$) was provided by Roth (Karlsruhe, Germany). Naringenin was from Sigma with a declared purity >95%. Water was purified through a Millipore Q-plus purification train system (Millipore, Bedford, MA, USA) and other solvents were of analytical grade.

2.3. Instrumentation

HPLC analyses were carried out on Shimadzu equipment (Shimadzu Corporation, Japan), consisting of liquid chromatograph LC-10AT, auto injector SIL-10A XL, column oven CTO-10A, a SPD-10A UV–VIS detector for the quantitative part and SPD-M10A diodearray detector for recording the UV-spectra in the range 200–400 nm. The column used was LiChroCART®, Superspher 100 RP-18 5 μm 125 × 4 mm i.d. from Merck (Darmstadt, Germany). The data collection and all calculations were performed by Shimadzu Class LC-10 software.

Stopped-flow absorption spectroscopy was performed as already described (Madsen et al., 1997) using a DM-17MV spectroflourometer from Applied Photophysics, London, UK. The reaction was followed at 25 °C by absorption measurements at 516 nm. The change in absorbance was analysed by non-linear regression analysis using the Pro/Kineticist software. Spectrophotometric data were acquired using a HP 8452A Diode Array Spectrophotometer (Hewlett-Packard Co., Palo Alto, CA, USA).

Table 2
Composed solutions of cichoric acid (two levels) and alkamides (three levels) for antioxidant assay

Code	Concentration, mg/ml	Compound	To be compared with
C1	0.30	Cichoric acid	All parts but leaves[a]
C2	1.80	Cichoric acid	Leaves
A1	0.02	Alkamides[b]	Side roots
A2	0.10	Alkamides[b]	Rootstock
A3	0.20	Alkamides[b]	Main root
C1A1	0.30	Cichoric acid	Side root
	0.02	Alkamides[b]	
C1A2	0.30	Cichoric acid	Rootstock
	0.10	Alkamides[b]	
C1A3	0.30	Cichoric acid	Main root
	0.20	Alkamides[b]	

The compositions are made to resemble extracts from different plant parts in the antioxidant assay. Antioxidant index determined are shown in Fig. 2.

[a] This is an average of the quantitative amount of cichoric acid in the four extracts.

[b] 'Alkamide mixture' as described in Section 2.

2.4. Method of extraction

Sample preparation. The dried plant material was minced in a coffee-grinder, and the powder kept in glass containers. Ground plant material, 400 mg, was sonicated with 10 ml methanol:water (70:30, v/v) for 30 min followed by 120 min of continuously mixing. The methanolic extract was centrifuged for 10 min (2000 rpm, $r = 0.15$ m). After extraction each sample was filtered through a 0.45 μm 13 mm filter and injected on HPLC for analysis. Results are means of duplicate chromatograms.

2.5. Purification and analysis of cichoric acid and alkamides

Cichoric acid was isolated from *Echinacea* fresh plant press juice powder (Paninkret®, Pinneberg, Denmark) (4 g) extracted with methanol–water (70:30) (40 ml, 30 min). The methanolic extract was centrifuged and the supernatant evaporated to remove methanol and water from the precipitate before redissolution in 50 ml of water. Preparative, reversed-phase HPLC (Discovery® RP 18, 21.2 × 250 mm, flow: 8 ml/min) gave cichoric acid (20 min, 254 nm detection). Final purification was controlled by

Table 1
Content of alkamides, cichoric acid and total phenolics in various plant parts and corresponding extracts of *E. purpurea*

Plant part	Alkamide 2, μg/g dry matter	Cichoric acid, mg/g dry matter	Alkamide 2, mg/ml extract	Total phenolics, mg/ml extract	Cichoric acid, mg/ml extract	Cichoric acid, % of total
Leaves	–	41.3	–	1.62	1.65	±100
Stem	–	8.57	–	0.30	0.34	±100
Rootstock	2.2	7.63	0.088	0.37	0.31	82
Main root	4.2	8.17	0.168	0.42	0.33	78
Side root	0.5	10.1	0.020	0.48	0.40	84

analytical HPLC, and the purity was found to be >95%. The alkamides used in the composed test samples were the natural mixture as isolated from the dried plants at the Danish University of Pharmaceutical Sciences, and identified by HPLC-MS. The quantification of total alkamides is an estimate based on an HPLC analysis of undeca-2Z,4E-dien-8,10-diynisobutylamid, (known as alkamide 2, cf. (Bauer & Wagner, 1990)) as described by Mølgaard, Johnsen, Christensen, and Cornett (2003). For analysis, the extract samples were mixed with the internal standard (0.4 mg/ml naringenin) and filtered through a 0.45 μm filter before injection to the analytical HPLC column at 40 °C. Three different eluents were used: A: acetonitrile/water 95:5, B: acetonitrile/water 5:95 and C: acetonitrile/water 5:95 added 0.1% trifluoroacetic acid (Mølgaard et al., 2003).

2.6. Colorimetric analyses for total phenolics

The content of total phenolics in the extracts was determined using the Folin–Ciocalteu method (Burns et al., 2000). Folin-Ciocalteu reagent (2.5 ml) was added to either 40 μl of extract or 40 μl of standard solutions to give 30 ml in total. After 8 min reaction time saturated sodium carbonate solution (7.5 ml) was added and the test solution was made up to 100 ml with H_2O and mixed. After 2 h the solutions were transferred to a cuvette and absorbance measured at 765 nm. Phenol was used as standard in concentrations 0.05, 0.10, 0.15, 0.25, 0.5, and 2.0 mg/ml, and the content of total phenolics in the extracts was calculated using linear regression (Perry, Burgess, & Glennie, 2001).

2.7. Measurement of antioxidant efficacy

Extracts and samples consisting of single solutions of cichoric acid and alkamides were used for the assays of antioxidant activity.

2.8. Oxygen consumption

Lipid oxidation (LH is an unsaturated lipid) is (1) initiated by metmyoglobin (Skibsted, Mikkelsen, & Bertelsen, 1998).

$$\mathrm{MbFe(III)} + \mathrm{LOOH} \rightarrow \mathrm{MbFe(II)} + \mathrm{LOO^{\cdot}} + \mathrm{H^{+}} \quad (1)$$

$$\mathrm{LOO^{\cdot}} + \mathrm{LH} \rightarrow \mathrm{L^{\cdot}} + \mathrm{LOOH} \quad (2)$$

$$\mathrm{L^{\cdot}} + \mathrm{O_2} \rightarrow \mathrm{LOO^{\cdot}} \quad (3)$$

$$\mathrm{LOO^{\cdot}} + \mathrm{AH} \rightarrow \mathrm{LOOH} + \mathrm{A^{\cdot}} \quad (4)$$

Oxygen consumption is characteristic for the propagation phase (reactions (2) and (3)) and the effect of added plant extract on the rate of depletion of oxygen in a peroxidating lipid emulsion is a direct measurement of the efficacy of the antioxidants on the progression of lipid oxidation. Chain-breaking antioxidants (AH) scavenge the chain-carrying lipid peroxyl radicals by donating a hydrogen atom (reaction (4)). The faster this reaction, the more efficient the antioxidant is. The oxygen concentration was measured with a Clark electrode using solutions saturated with air and oxygen-depleted solutions for calibration.

The oxygen consumption assay was carried out as reported by Hu and Skibsted (2002), using 30 μl of sample extract or solutions of cichoric acid or alkamides. The oxygen consumption was followed for 20 min, and the initial oxygen consumption rate $V(O_2)$ in $\mu mol\,l^{-1}\,s^{-1}$ was calculated from:

$$V(O_2) = \frac{-\mathrm{slope}[O_2]_{\mathrm{initial}} 10^6}{100}$$

The slope (percent O_2 per second) was calculated from the oxygen consumption in the 80–40% interval relative to the initial 100% oxygen concentration (water saturated with air). The influence of each of the extracts or solutions on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of extract (Hu & Skibsted, 2002):

$$I_{\mathrm{oxygen}} = 1 - \frac{V(O_2)\text{with extract present}}{V(O_2)\text{without extract present}}$$

The oxygen consumption assay is only a relative method for comparison of one compound or extract with another (Hu & Skibsted, 2002; Møller, Madsen, Aaltonen, & Skibsted, 1999). The index number has been reversed compared to our previous studies, and a higher value (close to unity) now indicates a high antioxidative effect.

2.9. Radical scavenging

Modifications were made to the original method of Brand-Williams, Cuvelier, and Berset (1995). The absorbance of 2.85 ml DPPH solution (0.040 mg DPPH/ml methanol) in quarts cuvettes was measured at 517 nm (maximum of DPPH) at 25 °C. Then 150 μl plant extract or cichoric acid in solution at similar concentration was added, and the absorbance was measured at 517 nm after 5 and 15 min to assure that the reaction had reached a steady state (Møller et al., 1999). DPPH is a purple coloured radical that, after being reduced by an antioxidant turns into a yellow product.

$$\mathrm{DPPH^{\bullet}(purple)} + \text{antioxidant} \rightarrow \text{Yellow non-radical product}$$

Cichoric acid (Fig. 1) has two ortho diphenols and each molecule is expected to react with four radicals to yield two quinones, i.e. the theoretical stoichiometric factor for radical scavenging would be four. The amount of DPPH reduced is calculated according to:

$$\mathrm{Abs_{DPPH-solution}} \cdot 0.95 - Abs_{+\mathrm{extract}} = \Delta\mathrm{Abs}$$

$$c = \Delta\mathrm{Abs} \cdot 3000(\text{volume of the cuvettes})/\varepsilon \cdot 150(\text{volume of extract}),$$

$$\varepsilon = 12,500\,\mathrm{l\,mol^{-1}\,cm^{-1}}$$

3. Results and discussion

3.1. Quantification

The content of active principles varies naturally between plant parts (Table 1). The concentration of cichoric acid is thus higher in extracts from leaves than from any other plant part. The aerial plant parts, i.e. stem and leaves, do not contain alkamides at all in contrast to the three different root extracts (Table 1). The concentrations in these and other Danish grown plants (Mølgaard et al., 2003) differ naturally from what is known from other European countries (Bauer & Wagner, 1990), although the range is of same magnitude. Compared to recent publications from Europe (Pellati et al., 2004; Becker et al., 2004) and from North America (Binns, Livesey, Arnason, & Baum, 2002) the root content of cichoric acid is relatively low, which is probably characteristic for the variety used in our investigation, i.e. Verbesserte Leuchtstern.

The natural variation in concentration of the constituents within the plant parts provides us with the opportunity to investigate the influence of the presence and absence of alkamides on the antioxidative effect of cichoric acid. For comparison with genuine extracts of *E. purpurea* plant material, we have composed simple mixtures of cichoric acid and alkamides at similar concentrations as those obtained in the plant extracts (Table 2).

3.2. Total phenolic content of plant extracts

It is interesting to notice the difference between above and below ground plant parts in respect of the relative content of plant phenolics. Apart from cichoric acid no phenolics are present in the leaf and stem extracts (Table 1). Cichoric acid accounts for 82%, 78% and 84% of the total phenolics in rootstock, main root and side roots, respectively. This is actually same proportion as determined by Pellati et al. (2004) leaving the remaining 17% to caftaric acid only. Beside that only minute peaks were present in the chromatogram (Mølgaard et al., 2003).

3.3. Antioxidant activity of extracts and isolated compounds

Fig. 2 shows the variation in antioxidant efficacy measured as antioxidative index values derived from the oxygen consumption rate for the different test solutions. The test samples (Table 2) are adjusted to concentrations similar to those determined in the different plant extracts, as shown in Table 1. The antioxidative index for solutions of the isolated compounds clearly show high activity of cichoric acid, and higher for C2 (1.8 mg/ml) than for C1 (0.3 mg/ml), indicating a dose–response dependence. The alkamides tested in three different concentrations (A1–A3, 0.2, 1.0 and 2.0 mg/ml) showed no antioxidative activity alone, rather were they prooxidative. However, in combination with the lowest concentration of cichoric acid, they increased the combined activity to a level approaching

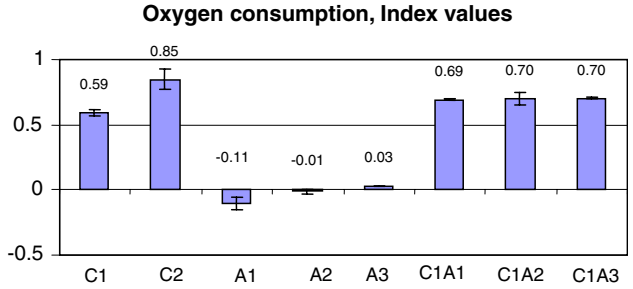


Fig. 2. Antioxidative index as measured in the oxygen consumption assay for a peroxidating lipid emulsion. Cichoric acid (C1, C2) and alkamides (A1, A2, and A3) were tested in the system individually and in combination (C1A1, C1A2, and C1A3). The concentrations of the different solutions and combinations are shown in Table 2. Negative values indicate a prooxidative effect.

that for the high concentration of cichoric acid alone (C2). The concentration of alkamides is probably too high to show a dose-response relation, but the results do indicate a synergistic effect of the alkamides in these mixtures (C1A1, C1A2, and C1A3). During the publication of this paper, we have been able to further confirm such synergistic effect in a LDL oxidation assay, including also activity of polysaccharides from the extract (Dalby Brown, Barsett, Landbo, Meyer, & Mølgaard, 2005).

Based on the structure of the alkamides no antioxidant activity is expected since no phenolic groups, aromatic rings or polyketide chains are present in the molecule. The observed improvement of the effect of cichoric acid mixed with alkamides can accordingly not be due to a direct radical scavenging, but rather to some surface activity of the alkamides giving the polar cichoric acid better access to inhibit lipid oxidation in the lipophilic droplets of the emulsion. The slight prooxidative effect of the alkamides alone in the lipid emulsion supports such a surface activity function. Synergism between the efficient antioxidant cichoric acid and the alkamide may also be based on another mechanism similar to what has been demonstrated for combinations of α-tocopherol and quercetin in the "water-like" solvent *tert*-butyl alcohol (Pedrielli & Skibsted, 2002). Quercetin, found to be an ineffective antioxidant in the hydrogen-bonding solvent, was capable of regenerating the efficient antioxidant α-tocopherol. This type of antioxidant synergism depending on regeneration of an efficient antioxidant by a less efficient was concluded to be of special importance for the water/lipid interphases. Alkamides could regenerate cichoric acid by donating an allylic hydrogen to the one-electron oxidized cichoric acid (Becker et al., 2004). Another possibility is that the alkamides may interact with the initiation step of lipid oxidation by reacting with metmyoglobin (reaction (1)).

The activity of cichoric acid was further confirmed when comparing the ability of the five different plant extracts to reduce the stable free radical DPPH. The antioxidative ability is clearly related to the concentration of cichoric acid (Table 3), since the leaf extract with the highest concentration of cichoric acid (1.62 mg/ml extract,

Table 3
Content of alkamide, cichoric acid and total phenolics in extracts in various part of *E. purpurea* compared with their ability to reduce DPPH

Plant part	Alkamide 2, mg/ml extract	Total phenolics, mg/ml extract	Cichoric acid, mg/ml extract	Cichoric acid, mg/ml in diluted extracts (5%)	Reduced DPPH, M	Stoichiometric factor[a]
Leaves	–	1.62	1.65	0.083	9.01E − 04	5.17
Stem	–	0.30	0.34	0.017	2.45E − 04	6.78
Rootstock	0.088	0.37	0.31	0.015	1.89E − 04	5.88
Main root	0.168	0.42	0.33	0.016	2.10E − 04	6.07
Side root	0.020	0.48	0.40	0.020	2.24E − 04	5.27

Cichoric acid, $M_W = 474.38$ g/mol.
[a] Example of calculation: 9.01E − 04 M/0.0825 mg/l · 474.38 g/mol = 5.17.

corresponding to 41.3 mg/g dry matter, Table 1) was by far the most efficient, reducing approximately four times the amount of DPPH compared to the other extracts. However, testing the five different extracts in the oxygen consumption assay (Table 4) did not reveal any substantial difference between plant parts, although the concentrations were very similar to previously tested extracts (Table 1). Each experiment must make use of freshly produced extracts, which explains minor differences in concentration values between Tables 1, 3 and 4. As the Index Values are not different in spite of different concentrations of cichoric acid, contribution to the antioxidative activity may come from constituents other than cichoric acid. DPPH has recently been demonstrated to provide accurate values for the stoichiometric factor for flavonoids as radical scavengers (Butkovíc, Klasinc, & Bors, 2004). The stoichiometric factor determined with DPPH (Table 3) has in the present study accordingly been used to estimate the contribution to the total activity of constituents other than cichoric acid. Since the theoretical value is expected to be four, and found to be 4.0 for isolated cichoric acid (Table 5), the higher values found (5.17–6.78) for extracts confirm a contribution also from compounds other than cichoric acid, which could be from caftaric acid (cf. (Pellati et al., 2004)), although only the root samples have high amounts of phenolics other than cichoric acid (Table 1).

Table 4
Oxygen consumption rate (Index value, *I*) compared with the content of cichoric acid in extracts of various parts of *E. purpurea*

Plant part	Cichoric acid, mg/g dry matter	Cichoric acid, mg/ml extract	Index value, I[a]
Leaves	47.3	1.89	0.87
Stem	9.5	0.38	0.82
Rootstock	7.0	0.28	0.86
Main root	8.8	0.35	0.76
Side root	11.0	0.44	0.83

New extracts of same raw material are made for each experiment, so values differ from those in Table 1.
[a] $I = 1 - (V_e/V_b)$, see text; V_e is velocity of sample, V_b is velocity of blank without extract added.

3.4. Comparison of cichoric acid with rosmarinic acid

Upon a comparison between cichoric acid and rosmarinic acid (Table 5), it is seen that their antioxidative activity is very similar in the oxygen consumption assay with an antioxidative index of 0.74 and 0.78, respectively, not significantly different ($p = 0.34$). Similar results are obtained in the radical scavenging assay with DPPH, with no significant difference between the two compounds ($p = 0.38$). As the molar concentration of cichoric acid is similar to that of rosmarinic acid in the assay (0.14 mM; Table 5) we may conclude that cichoric acid has an antioxidative activity comparable to that of rosmarinic acid, which is known to be an efficient antioxidant. In a comparison between different natural antioxidants, rosmarinic acid together with caffeic acid were found to be most efficient concerning what was a little imprecise termed antiradical power (ARP) as well as antioxidant power (AOP) (Brand-Williams et al., 1995).

3.5. Reaction kinetics

The oxygen consumption assay and the radical scavenging assay both identified cichoric acid as an efficient antioxidant. The kinetics of radical scavenging of cichoric acid was accordingly investigated using the stable radical DPPH and stopped-flow absorption spectroscopy. Cichoric acid

Table 5
Activity of cichoric acid compared to that of rosmarinic acid using the oxygen consumption and radical scavenging antioxidant assays

Active compound	Concentration 0.14 mM, mg/ml	Oxygen consumption assay, Index value[a]	Free radical method, reduced DPPH, M	Stoichiometric factor calculated from DPPH scavenging
Cichoric acid	0.060	0.74	5.53E − 04	4.0
Rosmarinic acid	0.050	0.78	6.26E − 04	4.5
		$p = 0.34$	$p = 0.38$	

[a] cf. Table 4 for calculation.

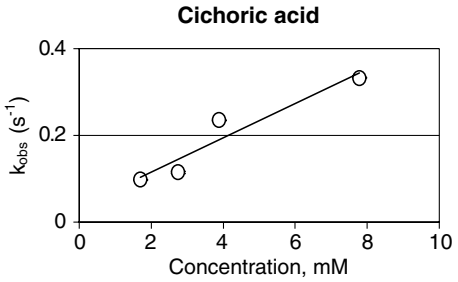


Fig. 3. The observed first-order rate constant for reduction of DPPH by cichoric acid in methanol at 25 °C measured by stopped flow absorption spectroscopy as function of the cichoric acid concentration.

was used in excess relative to DPPH, in order to establish pseudo-first order conditions. From the change in absorbance with time, the pseudo-first order constant, k_{obs}, was calculated for varying concentrations of DPPH, using non-linear regression analysis. In each case, the time course was well-represented by first order kinetics. A linear relationship between the concentration of cichoric acid and the observed pseudo-first order rate constant, k_{obs}, was confirmed:

$$k_{obs} = a + k_4[\text{cichoric acid}]$$

from which k_4 was estimated (Fig. 3) using linear regression, and in which the parameter a is a (non-significant) intercept. Similar second-order kinetics have recently been demonstrated for reaction of DPPH with series of flavonols and flavones in methanol (Butkovíc et al., 2004). The second-order rate constant k_4 for the reaction between DPPH and cichoric acid was found by linear regression to have the value 39.6 l/mol/s at 25 °C (Fig. 3) For comparison, Madsen, Møller Andersen, Viborg Jørgensen, and Skibsted (2000) found values for the flavonoids kaempferol and eriodictyol of 708 ± 72 and 33 ± 1 l/mol/s, respectively, for scavenging of DPPH. Cichoric acid may accordingly be concluded to be comparable with flavonoids as radical scavenger.

Our results have verified the antioxidative activity of the extract of *Echinacea purpurea*, and cichoric acid has been shown to account for the majority of this activity in accordance with Pellati et al. (2004). Cichoric acid is an efficient scavenger of free radicals and comparable to flavonoids as seen from the rate constant for reaction with the stable radical DPPH. For evaluation of the efficacy of *E. purpurea* extract in herbal medicine or functional foods we recommend standardisation based on cichoric acid. However, we have at the same time established that other constituents have a part to play. Although the alkamide fraction does not exhibit antioxidative activity on its own, it increases the activity of cichoric acid. Minor phenolics in the extract, e.g. caftaric acid, may also add to the activity (Pellati et al., 2004). We intend to investigate both the role of the phenols present in low concentrations and the mechanism behind the possible synergism between the alkamides and cichoric acid.

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