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Fenton reaction applied for screening natural antioxidants

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Abstract

Antioxidant activities of pure chemicals and food additives were compared with that of herb extracts using a method based on the Fenton reaction for screening natural antioxidants. This method detects antioxidants classified as free radical terminators, that may compete with linoleic acid for scavenging $^{\bullet}$OH radicals. Of the 39 products investigated, 19 were commercial products and 20 were aqueous and ethylic herb infusions. Commercial phenolic and flavonoid products, both hydrophilic and lipophilic, showed strong antioxidant activities, while indole, alkaloid and fs-cyclic products showed no antioxidant activity, as determined by this method. Aqueous infusions of all herbs showed antioxidant properties. Mild oregano, strong oregano, rosemary, sage and mint showed as strong antioxidant properties as pure commercial chemical and food additives, such as hydroquinone, rutin, phenidone, catechol, epicatechin, morin and BHA, in both aqueous and ethylic infusions. Also, the results suggest that, under the experimental conditions, the aqueous extractions of short duration yielded larger quantities of active compounds by reducing the degradation of their antioxidant properties.

Keywords: Fenton reaction; Natural antioxidants; Food additives; Herbs

1. Introduction

Oxidation by free radicals is an important event causing aging and human diseases, including cancer, multiple sclerosis, Parkinson's disease, autoimmune disease and senile dementia. After the absorption of food ingredients through intestinal and lung barriers and hepatic detoxification, the peroxidation of membrane lipids appears to be the starting point for cellular modifications (Appel, Roverts, & Woutersen, 1991; Aruoma, 1998; Feher, Csomos, & Vereckei, 1987; Halliwell & Gutteridge, 1989; Lessard, Lacroix, Ajdukovic, Charboneau, & Lamoureux, 1995). Stresses, physical damage, viral infection, cytotoxic or carcinogenic compounds, as a consequence of chemical or biological aggression, may cause peroxidation of cell membrane lipids and liberation of toxic substances, such as free radicals ($O_2^{\bullet -}$, $^{\bullet}$OH, and H$^{\bullet}$) (Aruoma, 1998; Czapski, 1984). Except in damaged tissue, cells have a very efficient antioxidant defence to counteract the toxic effects of free radicals. Superoxide dismutase (SOD) catalyzes the in vivo removal of superoxide anions ($O_2^{\bullet -}$) which are highly reactive in hydrophobic environments (Folkerth et al., 2004). Hydroxyl radicals ($^{\bullet}$OH) are the main free radicals present in vivo in an aqueous environment; they easily cross cell membranes at specific sites (Czapski, 1984). In biological systems, iron salts are always bound to proteins, membranes, nucleic acids, or low-molecular-weight chelating agents. They play a role in the biological redox system by ligand-linking to a number of extracellular protein antioxidants, such as transferrin and ceruloplasmin (Dumoulin, Chahine, Atanasiu, Nadeau, & Mateescu, 1996;

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Gutteridge, 1985; Osaki, Johnson, & Frieden, 1966). This reaction is important in the prevention of oxidative damage. Halliwell and Gutteridge (1985) developed an experimental system to evaluate lipid peroxidation by oxygen free radicals, for use in biological and medical research. This method is based on the Fenton reaction and detects non-enzymatic autoxidation. The iron(II)–EDTA complex in aqueous solution slowly autoxidizes to form $O_2^{\bullet -}$, which is rapidly dismutated to H_2O_2 at pH 7.4, and H_2O_2 interacts with iron(II), to form $^{\bullet}OH$ radicals by the Fenton reaction (1) in the presence of ascorbic acid as a catalyst (Gutteridge & Bannister, 1986; Kwon & Lee, 2004):

$$H_2O_2 + Fe^{2+}\text{–EDTA} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}\text{–EDTA} \quad (1)$$

$^{\bullet}OH$ free radicals attack unsaturated membrane lipids to form malonaldehyde (MDA), which may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen (Decker, Crum, & Calvert, 1992):

$$2\text{TBA} + \text{MDA} \rightarrow \text{chromogen} \quad (2)$$

The role of ascorbate is to reduce the iron(III) to iron(II) (3) and thus to favour the Fenton reaction:

$$Fe^{3+}\text{–EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{–EDTA} + \text{oxidized ascorbate} \quad (3)$$

Lipid oxidation is also a major cause of food quality deterioration (Labuza, 1996). A number of food additives and chemical products have been used in food preservation to prevent autoxidation (Dziezak, 1986). Although some synthetic antioxidants are very efficient in preventing autoxidation, only a few compounds are currently approved for use in the food industry. The major considerations for approval of such antioxidants are their potential toxicity and carcinogenicity. Thus, synthetic antioxidants continue to be scrutinized for their safety as food additives; consequently there is increasing public interest in the use of natural antioxidants. Plant tissues are rich in natural antioxidants (Zheng & Wang, 2001). Screening of plant antioxidants, and comparing their antioxidant potential with that of commercial food preservatives and synthetic products, will help find new sources of natural antioxidants (Wu, Lee, Ho, & Chang, 1982). Several methods have been used to measure the antioxidant properties (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Gutteridge & Bannister, 1986; Halliwell, Gutteridge, & Aruoma, 1987; Laughton, Halliwell, Evans, & Hoult, 1989; Le Tien, Vachon, Mateescu, & Lacroix, 2001; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004; Saint-Cricq de Gaulejac, Provost, & Vivas, 1999; Wang, Cao, & Prior, 1997). Non-enzymatic peroxidation of rat liver microsomes is one method for evaluating the ability of an antioxidant to inhibit oxidation and to prevent damage to cellular membranes (Quinlan, Halliwell, Moorhouse, & Gutteridge, 1988). This method is complex, expensive, due to the preparation of rat microsomes, and not suitable for multiple determinations of antioxidant activity. Wilbur, Bernheini, and Shapiro (1949) reported that linolenic acid (C18:3), both free and esterified in phospholipids, is an efficient scavenger of $^{\bullet}OH$ through lipid autoxidation with the formation of MDA. Linoleic acid (C18:2) is somewhat less efficient, but it is more stable in the light.

In order to obtain a more stable and reproducible system, artificial membranes were used in our study instead of rat liver microsomes, and the antioxidant properties of pure commercial products and of herb extracts were compared. The method allows the determination of the antioxidation potential of both hydrophilic and lipophilic compounds. Nineteen commercial products and 20 herbs were tested for their antioxidant properties.

2. Materials and methods

2.1. Reagents

Nineteen chemical or commercial products were used in our study. BHA, BHT, biochanin A, bathophenanthroline, catechol, gossypol, hydroquinone, indole-3-acetonitrile, morin, phytic acid, phenidone, rutin, vitamin A, glutathione, *N*-acetyl-L-cysteine and dithiothreitol were purchased from Sigma (St. Louis, MO, USA); ajmalicine, epicatechin and sempervirine were purchased from Indofine (Somerville, NJ, USA). Twenty herbs, listed in Table 1, were purchased from a local farmers market (Jean-Talon Market, Montreal, QC, Canada). All other chemicals used were obtained from Anachemia Inc. (Montreal, QC, Canada) and were of the highest purity available.

2.2. Liposomes preparation

Single bilayer phospholipid vesicles were formed by an injection method, as described by Batzri and Korn (1973)

Table 1
List of herbs studied

Common name	Botanical name
Anise	*Anethum graveolens* L.
Basil	*Ocimum basilicum* L.
Camomile	*Chamaemelum nobile* L.
Chives	*Allium schoenoprasum* L.
Celeriac	*Vallisneria alternifolia*
Common rue	*Ruta graveolens* L.
Coriander	*Coriandrum sativum* L.
Garlic	*Allium sativum*
Hibiscus	*Hibiscus abelmoschus* L.
Italian parsley	*Anthriscus cerefolium* L.
Marjoram	*Origanum marjorana* L.
Mint	*Mentha officinalis* L.
Parsley	*Petroselinum crispum*
Rosemary	*Rosemarinus officinalis* L.
Sage	*Salvia officinalis* L.
Savory	*Satureja hortensis*
Tarragon	*Artemisia dracunculus* L.
Thyme	*Thymus vulgaris* L.
Vervain	*Verbena officinalis* L.
Mild oregano, strong oregano	*Origanum vulgare* L. sp.

and Tyrrell et al. (1976). Lecithin from soybean, with linoleic acid as the main fatty acid moiety (Purity approx. 99%, Sigma, St. Louis, MO, USA), was dissolved in 3 ml of 95% ethanol. The ethanolic solution, containing 20–40 μmol of lecithin/ml was rapidly injected through a hypodermic syringe fitted with a fine needle (26 G) (Becton Dickinson Canada, Mississauga, ON, Canada) into phosphate buffer (20 mM, pH 7.4) maintained at room temperature in a proportion of 1:9 (v/v). All solutions had been purged of dissolved oxygen with nitrogen; chemical degradation is avoided and oxidation is controlled simply by working under nitrogen. The vesicles were concentrated to 3.5 ml at room temperature by filtration with rapid stirring under nitrogen pressure on an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, MA, USA), using an XM-100A membrane. Rapid stirring and low pressure were necessary to avoid formation of larger, more heterogeneous liposomes and the concentration of phosphatidylcholine in ethanol could not exceed 40 mM (Batzri & Korn, 1973). The liposomes were stable under a nitrogen atmosphere for several days at room temperature.

2.3. Liposomal lipid analysis

The fatty acid compositions of the liposomal lipids were analyzed by the method described by Yu, Kjellman, and Bjorksten (1996). Lipids were extracted 3 times with 1 ml of chloroform:methanol (1:2, v/v) from 5 ml of liposomes. The extracts were combined and the chloroform phase was evaporated to dryness under vacuum, and purified by the procedure of Bligh and Dyer (1959). The lipids were converted to their methyl esters according to the method of Slover and Lanza (1979). An aliquot of total lipid extract was treated with NaOH/MeOH and transmethylated with 14% (w/v) BF_3 in methanol. Methyl heneicosanoate (C21:0) was added as external standard. The fatty acid methyl esters were analyzed by capillary gas chromatography according to a method described by Mahrour, Caillet, Nketsia-Tabiri, and Lacroix (2003). A Varian gas chromatograph (Model 3400, Varian Associates Inc., Sunnyvale, CA, USA), equipped with a hydrogen flame ionization detector, Varian Star Chromatography Workstation software (1992) and a 30 m × 0.25 mm i.d., 1 μm film thickness DB-5 fused-silica capillary column (Supelco Inc., Oakville, ON, Canada) was employed. Helium was used as carrier gas. The split flow was set at 30 ml/min and the injector split ratio was adjusted to 50:1. The column temperature was held for 1 min at 80 °C and then increased at 20 °C/min to 150 °C and then at 4 °C/min to 280 °C. The injector temperature was increased from 70 to 300 °C at 100 °C/min and held for 60 min. The detector temperature was maintained at 300 °C.

2.4. Herb extraction

Herbs were purchased from a local supermarket (IGA, Laval, QC, Canada). Herbs were dried at room temperature and extracted through infusion with distilled water or ethanol at the concentration of 1 g of herb/10 ml. We compared extraction efficacies: an extraction of short duration (1 h for water and 4 h for ethanol) vs. an extraction of long duration (24 h for both water and ethanol). A 24 h period should allow extraction of compounds not easily extractable and, in particular, more phenolic compounds than when using an extraction of short duration. In the case of extraction of short duration, it was necessary to carry out an extraction for 4 h in ethanol, whereas an extraction of 1 h was sufficient, in water, to obtain similar quantities of extracts, sufficient for all experiments. The mixture was homogenized for 5 min under nitrogen (Sorvall Omni-Mixer, Mandel Scientific Co. Ltd., Guelph, ON, Canada) and macerated during 1 or 24 h in water, and 4 or 24 h in ethanol, respectively. The infusion was then filtered under vacuum, and the liquid was placed in a tube sealed with a screw-cap under nitrogen, and kept at −20 °C until used. For the determination of antioxidant activity, complete evaporation of water or ethanol extracts was done using the SpeedVac Automatic Evaporation system (Savant System, Holbrook, NY, USA). Each dried extract was weighed, then redissolved in distilled water or ethanol, to obtain known concentrations.

2.5. Commercial product preparation

The hydrophilic products (namely hydroquinone, phytic acid, rutin, glutathione, *N*-acetyl-L-cysteine and dithiothreitol) were dissolved in distilled water at the concentration of 3 mg/ml while the lipophilic products (i.e., 13 other products) were dissolved in chloromethane at the same concentration. These solutions were used for the determination of antioxidant activity after suitable dilution with the same solvents.

2.6. Determination of antioxidant activity (AA)

Antioxidant activity of herb extracts or solutions of commercial products was evaluated by a method based on the Fenton reaction, described by Halliwell and Gutteridge (1981) and Desmarchelier et al. (1998) but with modifications. This method detects antioxidants classified as free radical terminators, which may compete with linoleic acid to scavenge $^{\bullet}OH$ radicals.

The reaction mixture contained the following reagents at the final concentrations stated: 20 mM phosphate buffer (pH 7.4), 100 μM $FeCl_3$, 104 μM EDTA, 1 mM H_2O_2 and 100 μM sodium ascorbate. Solutions of $FeCl_3$ and sodium ascorbate were made up immediately before use in deaerated water. Measurement of the extent of liposomal lipid preoxidation was performed according to Menéndez, Amor, González, Jiménez, and Más (2000), using liposomes instead of rat liver microsomes. Liposomes, adjusted to a concentration of 10% (v/v) in the reaction mixture of 1 ml final volume, were immediately incubated, under agitation at 37 °C for 1 h, with 25 μl of herb extract solution or commercial product solution. Four final concentrations for the herb extracts (98, 391, 1563 and 6250 μg/ml) and the commercial products (4.9, 19.5, 78.1, 312.5 μg/ml) were tested. Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances (TBARS). After incubation, 1 ml of 1% (w/v) 2-thiobarbituric acid was then added, along with 1 ml of 2.8% (w/v) trichloroacetic acid. The mixture was vortexed for 3 min and sample tubes were transferred to an 80 °C water bath for 60 min to allow colour development, followed by cooling on ice. After extraction of the TBA chromophores with *n*-butanol and pyridine (15:1 v/v), the amount of TBARS formed was determined by reading the absorbance at 532 nm using a colorimeter (Microplate Autoreader EL 309, Bio-Tek Instruments, Winooski, VT, USA). A positive control was represented by the reaction mixture in the absence of sample, and the optical density of the chromogen formed denoted complete peroxidation. The negative control contained only the phosphate buffer without liposomes. The colorimetric reaction was calibrated with ascorbic acid for hydrophilic compounds and α-tocopherol for lipophilic compounds. The relative antioxidant activity was calculated using the following equation:

$$\begin{aligned}\mathrm{AA}\ (\%) = 1 - [&\mathrm{OD}_{(\text{negative control})} \\ &- \mathrm{OD}_{(\text{sample})}/\mathrm{OD}_{(\text{negative control})} \\ &- \mathrm{OD}_{(\text{positive control})}] \times 100.\end{aligned}$$

The following scale is proposed for the antioxidant activity percents (Laughton, Evans, Moroney, Hoult, & Halliwell, 1991): a product having an antioxidant activity of 70% was considered as a strong antioxidant; an antioxidant with an activity between 40% and 70% was considered as a medium strong antioxidant; an antioxidant with an activity less than 40% was considered as a neutral compound; a negative result indicates that the compound is pro-oxidant.

2.7. Statistical analysis

Analysis of variance and Duncan's multiple-range tests were employed to statistically analyze all results. Differences between means were considered significant when $P \leqslant 0.05$. Stat-Packets Statistical Analysis software (SPSS Base 10.0, SPSS Inc. Chigaco, IL, USA) was used for the analysis. For each measurement, three replicates in each sample were tested.

3. Results and discussion

Lecithin from soybeans is a mixture of phosphatides, mainly phosphatidylcholine, which contains polyunsaturated fatty acids (Schulz, Hansel, & Tyler, 1998). However its composition is variable from one commercial preparation to another. In our study, the liposomal fatty acid composition showed that polyunsaturated fatty acids were in a majority (data not shown), since the linoleic acid provided 61.6 ± 0.7% (w/w) and linolenic acid contributed 5.5 ± 0.1% (w/w). A high percentage of C18:2 and C18:3 from lecithin liposomes assured the system and should be efficient in replacing the rat liver microsomes, since the phospholipid bilayer of rat liver membranes is largely composed of polyunsaturated fatty acids. Quantities of polyunsaturated fatty acids in lipid bilayers of liver rat microsomes generally vary, according to the diet, between 45% and 55% of total fatty acids (Ulmann et al., 1991).

Results of the antioxidant activity of commercial products at four concentrations (4.9, 19.5, 78.1 and 312.5 μg/ml) and antioxidant strength at 313 μg/ml are presented in Table 2. The results showed that the antioxidant or pro-oxidant activity of commercial products increased with the concentration. Among hydrophilic compounds, hydroquinone (88.1%) and rutin (71.8%) were strong antioxidants in the liposome model system employed, with activities above 70%. Phytic acid (33.4%) was neutral as an antioxidant, while *N*-acetyl-L-cysteine (−92.2%), dithiothreitol (−97.9%) and glutathione (−112.1%) behaved as pro-oxidant products. With regard to the lipophilic compounds, phenidone (108.2%), catechol (96.8%), epicatechin (91.3%), morin (74.2%) and BHA (72.2%) were shown to be strong antioxidants. Gossypol (61.8%) and BHT (57.6%) showed medium antioxidant properties. Gossypol, is listed as "medium-strong" because its antioxidant activity was of medium strength when determined at 78.1 μg/ml. Due to an intrinsic colouring problem, its activity could not be evaluated at higher concentrations. Indole-3-acetonitrile (21.4%), vitamin A (15.7%) and ajmalicine (3.05%) were considered neutral. Sempervirine (−20.8%), biochanin A (−33.4%) and bathophenanthroline (−139.8%) showed pro-oxidant activity.

Tables 3 and 4 present the antioxidant activity and strength of aqueous and alcohol herb extracts, respectively. Four herb extracts concentrations (98, 391, 1563 and 6250 μg/ml) and two durations of extraction (1 and 4 h for aqueous herb extracts, and 4 and 24 h for alcohol herb extracts) were tested. In the case of the herb extracts, the relationship between the concentrations added to the liposome model system and the observed antioxidant or pro-oxidant activity was less obvious than it was with the commercial products. Indeed, the antioxidant activities of several extracts did not seem to be affected by the concentration; this was the case, in particular, for garlic, celeriac, coriander and common rue after extraction for 24 h with water, and for celeriac and parsley after extraction for 4 h with ethanol. The extracts from mild oregano (78.9%), rosemary (76.6%) and sage (70.4%) after a 1 h aqueous extraction, strong oregano (77.9% in water 24 h and 90.5% in ethanol 4 h), mint (71.7% in ethanol 4 h) and sage (79.7% and 74.4% in ethanol 4 and 24 h, respectively) showed strong antioxidant activities. Garlic (42.6%), mint (43.8%), thyme (41.1%) and basil (68.4%), after 1 h aqueous extraction, rosemary (51.3%) and marjoram (50.1%) after 24 h aqueous extractions, rosemary

Table 2
Antioxidant activity and strength of commercial products

Solubility	Products	Chemical family	Antioxidant activity (%, mean ± SD)[a]				Antioxidant strength at 313 μg/ml
			4.9 μg/ml	19.5 μg/ml	78.1 μg/ml	313 μg/ml	
Hydrophilic	Hydroquinone	Phenoid	39.1 ± 2.8Aij	77.3 ± 4.4Bm	89.4 ± 4.1Cq	88.1 ± 2.8Cm	Strong
	Rutin	Flavonoid	33.3 ± 3.2Ai	38.2 ± 2.5Aj	57.3 ± 2.2Bm	71.8 ± 1.7Cl	Strong
	Phytic acid	Sugar phosphate	20.1 ± 1.1Ah	25.2 ± 2.9Bh	28.5 ± 1.3Bj	33.4 ± 1.1Cj	Neutral
	Glutathione	Aminothiol	4.2 ± 1.9Dd	−15.5 ± 0.3Cc	−54.3 ± 1.5Bb	−112.1 ± 3.3Ab	Pro-oxidant
	N-Acetyl-L-cysteine	Aminothiol	3.1 ± 2.6Dd	−6.1 ± 1.0Cd	−15.3 ± 1.5Bd	−92.2 ± 0.8Ad	Pro-oxidant
	Dithiothreitol	Thiol	1.1 ± 1.1Dcd	−19.0 ± 1.1Cb	−58.9 ± 2.1Ba	−97.9 ± 1.5Ac	Pro-oxidant
Lipophilic	Phenidone	Phenylamine derivative	11.1 ± 0.3Ae	25.3 ± 2.3Bh	80.7 ± 2.3Cp	108 ± 7.5Do	Strong
	Catechol	Flavonoid	41.7 ± 1.3Aj	68.3 ± 3.4Bl	92.2 ± 3.5Cq	96.8 ± 4.2Cno	Strong
	Epicatechin	Flavonoid	14.4 ± 0.4Af	37.2 ± 1.5Bj	60.3 ± 1.8Cn	91.3 ± 2.4Dmn	Strong
	Morin	Flavonoid	10.7 ± 1.4Ae	14.7 ± 2.3Bf	43.5 ± 1.0Ck	74.2 ± 1.1Dl	Strong
	BHA	Phenol derivative	17.2 ± 0.7Ag	32.4 ± 2.4Bi	69.7 ± 1.4Co	72.2 ± 2.4Cl	Strong
	BHT	Phenol derivative	19.5 ± 1.9Agh	24.3 ± 1.6Bh	47.8 ± 0.8Cl	57.6 ± 2.9Dk	Medium
	Gossypol	Naphthyl derivative	38.1 ± 3.4Aij	51.6 ± 4.1Bk	61.8 ± 1.3Cn	ND[b]	Medium strong[c]
	Indol-3-acetonitrile	Indol	−13.7 ± 0.7Aa	−3.0 ± 0.5Be	−7.1 ± 0.7Cf	21.4 ± 0.4Di	Neutral
	Vitamin A	Carotenoid	−7.8 ± 0.8Ab	−4.4 ± 1.6Bde	19.0 ± 1.7Ci	15.7 ± 1.6Ch	Neutral
	Ajmalicine	Alkaloid	−7.6 ± 1.0Ab	−6.2 ± 0.3Ad	4.1 ± 0.9Bh	3.0 ± 0.5Bg	Neutral
	Biochanin A	Isoflavone	−1.8 ± 1.3Dc	−23.1 ± 1.9Ca	−28.3 ± 1.6Bc	−33.4 ± 0.8Ae	Pro-oxidant
	Sempervirine	Alkaloid	0.2 ± 0.5Cc	−13.3 ± 1.7Bc	−10.0 ± 0.9Be	−20.8 ± 2.3Af	Pro-oxidant
	Bathophenanthroline	Aza-phenanthrene	21.3 ± 2.1Ch	18.3 ± 0.9Cg	1.2 ± 1.1Bg	−140 ± 5.2Aa	Pro-oxidant

[a] Means in the same row bearing the same uppercase letter are not significantly different ($P > 0.05$). Means in each column bearing the same lowercase letter are not significantly different ($P > 0.05$).

[b] ND: Not determined (colouring problem).

[c] Strength determined at 78.1 μg/ml.

(66.3% and 49.7% in ethanol 4 and 24 h, respectively) and thyme (54.4% and 51.1% in ethanol 4 and 24 h, respectively), mint (71.7%), mild oregano (41.2%) and strong oregano (49.3%) after a 24 h ethanol extraction, showed a medium antioxidant activity. The other extracts were neutral with respect to antioxidant or pro-oxidant activity under our experimental conditions.

Based on the experimental conditions employed, the majority of herbs studied showed the best antioxidant activity at a concentration of 6.25 mg/ml. Table 5 summarizes the extraction conditions, yielding the strongest antioxidant activity at 6.25 mg/ml, for each herb. This table takes into account type of solvent, extraction time, antioxidant activity and strength. The majority of the herbs studied showed best antioxidant activity when extracted in distilled water. For 15 extracts (both water and ethanol), antioxidant activities were higher for the 1 and 4 h extractions than for the 24 h extractions. One hour extraction in water apparently offers the best preservation of the antioxidant properties for 11 extracts. Camomile, savory, marjoram and tarragon yielded the strongest antioxidant activities after extraction for 24 h in water. Mint, sage, strong oregano and thyme showed the best antioxidant activities after extraction for 4 h in ethanol. Common rue yielded the highest antioxidant activity after extraction for 24 h with ethanol.

Twelve of the products investigated yielded strong antioxidant activity and 6 products showed medium antioxidant properties. These products were chosen from the literature as having antioxidant or other biological properties beneficial to cells susceptible to carcinogenic transformation (Appel et al., 1991; Chae et al., 1991; Colacchio, Memoli, & Hildebrandt, 1989; Laughton et al., 1991; Singletary & Nelshoppen, 1991a, 1991b; Stahelin et al., 1991; Szmurlo et al., 1991; Zhang, Cooney, & Bertram, 1991; Zheng & Wang, 2001).

Nineteen of the products selected were pure commercial compounds, which allowed comparison of their properties within the same family of chemical compounds. All studied flavonoids (namely catechol, epicatechin, rutin and morin) showed strong antioxidant activities. The results obtained with morin (pentahydroxyflavone) and the rutin (pentahydroxyflavone-3-rutinoside) are similar to the results reported by Laughton et al. (1991). These authors listed gossypol as a strong antioxidant, although it showed medium antioxidant activity at 78.1 μg/ml under our experimental conditions. Its red colouring, relatively strong at 313 μg/ml, may have interfered during the colorimetric readings. Phenoid and phenylamine derivatives were also considered to be strong antioxidants. Flavonoids are a group of natural benzo-γ-pyran derivatives and occur as aglycones, glycosides and methylated derivatives. The key role of flavonoids, as scavengers of free radicals, is emphasized in several reports (Laughton et al., 1989; Saint-Cricq de Gaulejac et al., 1999; Stavric, Matula, Klassen, & Downie, 1996; Wang et al., 1997). Antioxidant activity is dependent on the structure of the free radical-scavenging compounds and the substituents present on the ring of

Table 3
Antioxidant activity and strength of aqueous extracts of herbs

Extracts	Antioxidant activity (%, mean ± SD)[a]								Antioxidant strength at 6250 µg/ml	
	One-hour extraction				Twenty four-hour extraction				One hour	Twenty four hours
	98 µg/ml	391 µg/ml	1563 µg/ml	6250 µg/ml	98 µg/ml	391 µg/ml	1563 µg/ml	6250 µg/ml		
Anise	21.3 ± 2.1Ag	28.6 ± 1.3Bf	45.4 ± 3.3Cf	27.4 ± 1.4Bc	ND[b]	ND	ND	ND	Neutral	ND
Basil	21.6 ± 1.2Ag	39.7 ± 1.0Bh	56.8 ± 2.6Ch	68.4 ± 3.2Df	ND	ND	ND	ND	Medium	ND
Camomile	ND	ND	ND	ND	24.4 ± 1.4Bg	21.4 ± 2.3Bh	25.5 ± 1.8Be	6.4 ± 1.9Aa	ND	Neutral
Chives	1.1 ± 0.6Bd	16.8 ± 0.6Dd	33.2 ± 1.7Fd	37.2 ± 2.2Fd	−3.3 ± 0.3Ac	6.2 ± 0.5Ce	20.6 ± 2.5Ecd	20.3 ± 1.3Ede	Neutral	Neutral
Celeriac	31.4 ± 1.8Ah	35.2 ± 2.8ABg	34.2 ± 0.9ABd	35.3 ± 2.4ABd	31.1 ± 2.8Ahi	37.1 ± 1.2Bj	31.3 ± 2.0Ag	ND	Neutral	ND
Common rue	ND	ND	ND	ND	22.4 ± 1.5Bg	15.4 ± 1.7Ag	23.0 ± 2.7Bde	17.4 ± 1.6Acd	ND	Neutral
Coriander	4.4 ± 0.9Ae	10.3 ± 1.2Bc	15.2 ± 0.9Da	16.0 ± 1.3Db	17.2 ± 2.2Df	14.4 ± 1.2CDg	24.1 ± 2.2Ede	11.7 ± 1.3BCb	Neutral	Neutral
Garlic	27.2 ± 2.8Ch	32.5 ± 2.6Cg	38.6 ± 1.3De	42.6 ± 1.1Ee	16.7 ± 1.1Bf	9.1 ± 0.8Af	16.9 ± 1.3Bc	19.6 ± 1.7Bde	Medium	Neutral
Italian parsley	−1.7 ± 1.6Bc	0.3 ± 1.5BCa	13.3 ± 1.1Ea	19.3 ± 2.0Fb	3.2 ± 1.4Cd	−5.4 ± 0.9Ac	7.1 ± 0.7Db	18.7 ± 2.8Fcde	Neutral	Neutral
Marjoram	ND	ND	ND	ND	21.7 ± 1.7Ag	30.5 ± 1.3Bi	46.7 ± 4.1Ci	50.1 ± 1.7Ch	ND	Medium
Mint	0.2 ± 0.8Bcd	9.0 ± 0.7Dc	24.4 ± 1.4Ec	43.8 ± 1.8Fe	−7.8 ± 1.6Ab	−9.1 ± 0.7Ab	5.6 ± 1.5Cab	4.1 ± 1.1Ca	Medium	Neutral
Parsley	−9.4 ± 1.9Bb	−2.3 ± 1.2Ca	21.3 ± 0.9Eb	35.3 ± 1.7Fd	−17.6 ± 1.2Aa	−13.6 ± 2.5ABa	3.4 ± 0.8Da	23.3 ± 2.5Ee	Neutral	Neutral
Rosemary	−15.4 ± 1.7Aa	3.2 ± 0.5Cb	49.5 ± 2.5Efg	76.6 ± 1.4Fg	−4.8 ± 1.6Bbc	−1.6 ± 1.6Bd	34.2 ± 1.4Dg	51.3 ± 2.7Eh	Strong	Medium
Sage	5.7 ± 0.7Ae	22.7 ± 1.7Be	45.8 ± 2.1Cf	70.4 ± 2.5Df	ND	ND	ND	ND	Strong	ND
Savory	21.5 ± 2.1Cg	25.9 ± 2.4CDef	26.8 ± 1.8Dc	11.0 ± 1.3Aa	17.1 ± 1.3Bf	23.4 ± 2.3CDh	32.5 ± 1.2Eg	35.4 ± 3.4Efg	Neutral	Neutral
Tarragon	ND	ND	ND	ND	35.6 ± 3.1Ci	27.5 ± 2.0Bi	37.3 ± 1.6Ch	21.6 ± 1.7Ae	ND	Neutral
Thyme	20.1 ± 2.1Ag	40.8 ± 1.9CDh	51.6 ± 1.8Fg	41.1 ± 1.6De	28.3 ± 1.3Bh	35.4 ± 3.6Cj	45.3 ± 1.9Ei	39.7 ± 1.9CDg	Medium	Neutral
Vervain	10.1 ± 1.6Cf	19.6 ± 2.4DEde	23.3 ± 1.6EFbc	24.5 ± 1.7Fc	7.2 ± 1.4BCe	−4.6 ± 1.5Acd	6.7 ± 0.6Bb	16.1 ± 1.2Dc	Neutral	Neutral
Mild oregano	9.9 ± 0.4Bf	51.2 ± 2.2Di	62.1 ± 3.0Eh	78.9 ± 1.8Fg	3.0 ± 0.5Ad	10.2 ± 1.7Bf	28.5 ± 0.4Cf	30.7 ± 3.4Cf	Strong	Neutral
Strong oregano	ND	ND	ND	ND	32.3 ± 2.2Ai	39.3 ± 2.9Bj	68.2 ± 4.1Cj	77.9 ± 1.9Di	ND	Strong

[a] Means in the same row bearing the same uppercase letter are not significantly different ($P > 0.05$). Means in each column bearing the same lowercase letter are not significantly different ($P > 0.05$).

[b] ND: Not determined.

Table 4
Antioxidant activity and strength of ethanol extracts of herbs

Extracts	Antioxidant activity (%, mean ± SD)[a]								Antioxidant strength at 6250 μg/ml	
	Four-hour extraction				Twenty four-hour extraction				Four hours	Twenty four hours
	98 μg/ml	391 μg/ml	1563 μg/ml	6250 μg/ml	98 μg/ml	391 μg/ml	1563 μg/ml	6250 μg/ml		
Anise	18.9 ± 1.1Hgh	2.5 ± 1.4Fe	−14.4 ± 1.1Ce	−32.5 ± 2.4Bd	10.3 ± 1.2Gd	−7.2 ± 0.3Ec	−10.4 ± 1.3Dc	−40.7 ± 2.8Ac	PO[b]	PO
Basil	20.2 ± 1.2Ch	11.3 ± 0.8Bf	12.1 ± 1.1Bg	10.4 ± 1.5Bfg	21.1 ± 0.7Cf	11.2 ± 0.7Be	12.7 ± 1.8Bf	5.1 ± 1.0Ae	Neutral	Neutral
Camomile	−33.6 ± 2.1Db	−65.9 ± 2.3Cb	−66.7 ± 1.4Ca	−199.8 ± 4.3Aa	−11.6 ± 0.8Fc	−12.3 ± 0.9Fb	−17.8 ± 1.7Eb	−126.3 ± 6.6Ba	PO	PO
Chives	−8.7 ± 1.0Ae	5.0 ± 1.1Be	23.9 ± 1.5Dh	12.5 ± 1.7Cgh	30.4 ± 2.5Egh	23.6 ± 0.9Dhi	35.4 ± 3.3Ei	15.4 ± 1.8Cg	Neutral	Neutral
Celeriac	17.7 ± 1.2Dg	10.5 ± 0.3Cf	7.2 ± 0.5Bf	15.2 ± 1.9Dh	9.7 ± 0.8Cd	15.1 ± 1.5Df	24.2 ± 2.7Eh	4.3 ± 0.3Ae	Neutral	Neutral
Common rue	ND[c]	ND	ND	ND	15.8 ± 1.9Ae	19.7 ± 2.0Ag	40.4 ± 0.9Cj	29.8 ± 0.8Bh	ND	Neutral
Coriander	−56.2 ± 2.3Aa	−7.3 ± 0.5Dd	−12.2 ± 1.2Ce	−26.4 ± 1.3Be	10.1 ± 0.8Ed	25.3 ± 1.3Fi	23.7 ± 2.1Fh	26.8 ± 2.2Fh	PO	Neutral
Garlic	−10.9 ± 1.3Ae	11.1 ± 0.7Bf	25.2 ± 1.7Ch	32.4 ± 2.4DEj	31.2 ± 1.7Dh	27.4 ± 2.0Ci	35.4 ± 2.2Ei	28.7 ± 1.9CDh	Neutral	Neutral
Italian parsley	11.2 ± 1.4Bf	11.6 ± 0.9Bf	23.5 ± 1.3Dh	13.4 ± 1.8BCgh	15.4 ± 1.2Ce	2.2 ± 1.6Ad	5.0 ± 1.4Ae	10.9 ± 1.4Bf	Neutral	Neutral
Marjoram	−25.2 ± 1.4Ac	14.2 ± 1.0Dg	26.5 ± 2.6Eh	8.3 ± 0.9Cf	−13.4 ± 1.1Bc	5.6 ± 1.9Cd	17.1 ± 1.9Dg	41.5 ± 0.8Fi	Neutral	Medium
Mint	38.1 ± 2.6Cj	41.0 ± 1.8Ch	59.5 ± 0.7Ei	71.7 ± 2.7Fm	27.1 ± 1.3Ag	31.1 ± 0.4Bj	54.4 ± 0.4Dk	59.3 ± 2.1Ek	Strong	Medium
Parsley	13.1 ± 1.2Bf	10.7 ± 1.6ABf	12.9 ± 0.6Bg	13.1 ± 1.3Bgh	9.1 ± 0.3Ad	21.2 ± 1.5Cgh	12.5 ± 1.3Bf	8.2 ± 1.5Af	Neutral	Neutral
Rosemary	29.2 ± 2.0Ai	39.9 ± 1.1Bh	58.8 ± 2.5Di	66.3 ± 2.0El	32.3 ± 2.1Ah	45.4 ± 1.8Ck	59.6 ± 2.7Dl	49.7 ± 2.7Cj	Medium	Medium
Sage	38.4 ± 2.6Aj	59.8 ± 2.3Cj	74.7 ± 3.5Dk	79.7 ± 2.1Dn	45.2 ± 2.5Bi	62.5 ± 2.8Cm	75.0 ± 4.4Dm	74.4 ± 3.2Dl	Strong	Strong
Savory	−20.4 ± 0.4Cd	−6.2 ± 1.2Ed	−31.3 ± 0.4Bd	−43.1 ± 2.7Ac	−18.9 ± 2.7CDb	−15.4 ± 0.6Da	20.0 ± 1.9Ggh	5.3 ± 1.0Fe	PO	Neutral
Tarragon	−53.6 ± 2.8Ba	−39.4 ± 1.0Cc	−40.2 ± 1.4Cc	−101.1 ± 3.6Ab	−12.8 ± 1.3Fc	−16.4 ± 0.8Ea	−31.2 ± 0.4Da	−56.5 ± 1.2Bb	PO	PO
Thyme	13.3 ± 0.8Af	54.1 ± 2.1Bi	65.3 ± 1.6Cj	54.4 ± 2.4Bk	50.7 ± 2.3Bj	54.5 ± 2.9Bl	64.2 ± 3.5Cl	51.1 ± 1.9Bj	Medium	Medium
Vervain	−57.2 ± 3.1Ca	−96.6 ± 3.6Ba	−50.2 ± 2.3Db	−106.5 ± 5.3Ab	−37.9 ± 2.2Ea	−13.9 ± 1.8Gab	−4.12 ± 0.5Hd	−25.8 ± 1.3Fd	PO	PO
Mild oregano	12.6 ± 0.4Bf	16.7 ± 1.8Cg	23.1 ± 1.0Dh	19.6 ± 1.7Ci	13.1 ± 1.1Be	5.3 ± 1.7Ad	17.3 ± 1.3Cg	41.2 ± 2.8Ei	Neutral	Medium
Strong oregano	27.6 ± 1.1Ai	39.4 ± 1.3 Bh	77.5 ± 4.6Fk	90.5 ± 1.3Go	54.9 ± 2.6Dj	63.6 ± 3.4Em	60.4 ± 2.3El	49.3 ± 1.8Cj	Strong	Medium

[a] Means in the same row bearing the same uppercase letter are not significantly different ($P > 0.05$). Means in each column bearing the same lowercase letter are not significantly different ($P > 0.05$).

[b] PO: Pro-oxidant.

[c] ND: Not determined.

Table 5
Conditions of extraction to obtain the highest antioxidant activity of herb at 6250 μg/ml

Extracts	Solvent	Extraction time (h)	Antioxidant activity (%, mean ± SD) at 6250 μg/ml	Antioxidant strength at 6250 μg/ml
Anise	Water	1	27.4 ± 1.4	Neutral
Basil	Water	1	68.4 ± 3.2	Medium
Camomile	Water	24	6.4 ± 1.9	Neutral
Chives	Water	1	37.2 ± 2.2	Neutral
Celeriac	Water	1	35.3 ± 2.4	Neutral
Common rue	Ethanol	24	29.8 ± 0.8	Neutral
Coriander	Water	1	16.0 ± 1.3	Neutral
Garlic	Water	1	42.6 ± 1.1	Medium
Italian parsley	Water	1	19.3 ± 2.0	Neutral
Marjoram	Water	24	50.1 ± 1.7	Medium
Mint	Ethanol	4	71.7 ± 2.7	Strong
Mild oregano	Water	1	78.9 ± 1.8	Strong
Parsley	Water	1	35.3 ± 1.7	Neutral
Rosemary	Water	1	76.6 ± 1.4	Strong
Savory	Water	24	35.4 ± 3.4	Neutral
Sage	Ethanol	4	79.7 ± 2.1	Strong
Strong oregano	Ethanol	4	90.5 ± 1.3	Strong
Tarragon	Water	24	21.6 ± 1.7	Neutral
Thyme	Ethanol	4	54.4 ± 2.4	Medium
Vervain	Water	1	24.5 ± 1.7	Neutral

the flavonoids (Chen, Chan, Ho, Fung, & Wang, 1999). The spatial arrangement of substituents is a greater determinant of antioxidant activity than the flavan backbone alone (Heim, Tagliaferro, & Bobilya, 2002; Rice-Evans, Miller, & Paganga, 1996). Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity (Burda & Oleszek, 2001; Sekher Pannala, Chan, O'Brien, & Rice-Evans, 2001). Antioxidant activity is primarily attributed to the high reactivities of hydroxyl substituents. The B-ring hydroxyl configuration is the most significant determinant of scavenging of reactive oxygen species (Burda & Oleszek, 2001). Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. There is an increasing interest in the biological effects of flavonoids. These important compounds form an integral part of human diet, and could be helpful against human cancers, arteriosclerosis, ischaemia events and inflammatory disease, which are partially caused by exposure to oxidative stress (Halliwell, 1996; Namiki, 1990). Several flavonoids have been reported to quench active oxygen species and inhibit in vitro oxidation of low-density lipoproteins and therefore reduce thrombic tendency (Frankel, German, Kinsella, Parks, & Kanner, 1993). In addition, flavonoids may offer an alternative way to protect lipids from oxidation in foods. Some of these flavonoids have been shown to inhibit oxidation in meats, fish oil, lard and sunflower oil (Chen et al., 1999; Ŝkerget et al., 2005).

The two commonly used synthetic food additives, BHA and BHT, showed different antioxidant potentials, despite being chemically similar. Although BHA is very efficient in preventing autoxidation, this compound is currently less and less used in the food industry, with the profit of natural antioxidant compounds. BHA and BHT have been suspected to cause or promote negative health effects (Barlow, 1990; Namiki, 1990; Pokorný, 1991). For this reason, there is a growing interest in the antioxidant properties of the antioxidant compounds contained in plants which derive from their strong activity and low toxicity compared with those of synthetic phenolic antioxidants, such as BHT and BHA (Marinova & Yanishlieva, 1997; Nakatani, 1996).

All thiols (aminothiols and thiols) studied, which belong to the same family of chemicals, showed pro-oxidant properties. However, De Flora, Izzotti, D'Agostini, and Cesarone (1991) reported that thiols, such as glutathione, *N*-acetyl-L-cysteine and dithiothreitol, possess anti-cancer potential activity. These compounds apparently reduce the damage caused to DNA by X-rays or 2-acetylaminofluorene. This suggests that some compounds may also contribute to the prevention of cancer through mechanisms other than their pro-oxidant properties. Antioxidant potential is thus one among several mechanisms that prevent cancer.

Our study showed that, of the 20 tested herb extracts, the mild and strong oregano, sage, rosemary and mint were the most effective. The results of antioxidant activity obtained for herb extracts are comparable with those presented in the literature (Cuvelier, Richard, & Berset, 1996; Economou, Oreopoulou, & Thomopoulos, 1991; Oussalah et al., 2004; Zheng & Wang, 2001) which indicate that oregano, sage and rosemary have strong antioxidant activities. The antioxidant activity of herbs and spices is caused mainly by phenolic compounds, such as flavonoids, phenolic acids and phenolic monoterpenes (Radonic & Milos, 2003; Rice-Evans et al., 1996; Zheng & Wang, 2001). Oregano has been extensively studied as an effective antioxidant in the lipid system (Lagouri & Boskou, 1996) and its antioxidant activity was higher than that of α-tocopherol and comparable to that of BHA against linoleic acid oxidation (Nakatani, 1996). Oregano species extracts had high contents of rosmarinic acid, carvacrol, thymol and hydroxycinnamic acid (Radonic & Milos, 2003; Zheng & Wang, 2001) and these compounds have been demonstrated to possess strong antioxidant activity (Aeschbach et al., 1994; Burits & Bucar, 2000; Chen & Ho, 1997). The principal phenolic compounds of rosemary are rosmanol, rosmarinic acid, naringin and carnosic acid (Zheng & Wang, 2001). Cuvelier et al. (1996) measured the correlation between antioxidant efficiency and the composition of sage and recognized that carnosol, rosmarinic acid, and carnosic acid had the greatest antioxidant activities, followed by caffeic acid and cirsimaritin. In addition, many volatile constituents of sage, such as 1,8-cineole, thujone and camphor, contribute to the antioxidant properties (Zheng & Wang, 2001). Concentrations used to study the antioxidant activity of some herb extracts were approximately 20 times higher than those of commercial products. These high concentrations are justified because the filtrates collected from the herbs were crude extracts without purification, while commercial products were highly purified. Once purified, micronutrients with antioxidant properties present in those extracts will likely be equal or superior to the positive controls when used at similar concentrations.

Aqueous extraction of short duration yielded the highest antioxidant values. Of the fifteen 1-h aqueous extraction, three showed a strong antioxidant activity (mild oregano, rosemary and sage), four showed a medium antioxidant activity (basil, garlic, mint and thyme) and eight were neutral. In comparison, from the sixteen 24-h aqueous extractions, one was strongly antioxidant (strong oregano), two were of medium strength (marjoram, rosemary) and thirteen were neutral. These results suggest that, under our experimental conditions, the aqueous extractions of short duration are preferable for recovery and preservation of the antioxidant properties of micronutrients contained in herbs. Aqueous extracts that were infused for a longer period lost their antioxidant properties, possibly due to chemical degradation or to long-term oxidation. With regard to ethanol extraction, three (strong oregano, mint and sage) and one (sage) extracts showed a strong antioxidant activity after a 4 h extraction and a 24 h extraction, respectively. The antioxidant activities of several herbs obtained in aqueous extracts differ from those obtained in ethanol extracts. These results indicate that the phenols obtained in aqueous extracts have antioxidant activities which are more significant than those of phenols obtained in ethanol extracts. The reason is certainly the variation of solubility of compounds extracted in water or ethanol, which is connected to their hydrophilic or hydrophobic character. According to these results, it appears that the polarity of phenolic compounds is a determinant of antioxidant activity. The polarity of the flavonoids depends primarily on the nature of the radicals on rings, and in particular on the number of OH groups (Heim et al., 2002; Vasserot, Caillet, & Maujean, 1997). The differences in antioxidant activity between polyhydroxylated and polymethoxylated flavonoids are most likely due to differences in both hydrophobicity and molecular planarity (Heim et al., 2002).

4. Conclusion

The simple technique for measuring antioxidant activity, which was developed using lecithin liposomes, produced results in accordance with literature findings. A strong antioxidant activity was measured for compounds such as hydroquinone, flavonoids (rutin, morin and epicatechin), BHA and some herbs (oregano, sage and rosemary). Our results suggest that, under the experimental conditions employed in this study, the aqueous extractions of short duration (one hour), are generally better for recovery and preservation of antioxidant properties of the herb compounds studied. It would therefore be advantageous to add herbs to foods at the end of cooking in order to maintain their flavour and to preserve their biological properties. Micronutrients are digested before being absorbed. The solubility of these products is not of prime concern in an in vivo biological context. However, it is important for in vitro studies aiming at assessing their antioxidant potential. It should be noted that, even if a product lacks antioxidant properties, it may still possess other biological properties important for the defence mechanisms of the cells against chemical or biological aggression. This paper presents an initial selection of herbs. It is desirable to extend these studies, and to further characterize the most active fractions.

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