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Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies

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Abstract

Ethanolic extracts from the roots of wild licorice (*Glycyrrhiza lepidota*), narrow-leaved echinacea (*Echinacea angustifolia*), senega (*Polygala senega*), leaves of bearberry (*Arctostaphylos uva-ursi*) and aerial parts of two varieties of horsetail (*Equisetum* spp.) were prepared and evaluated for their free-radical scavenging capacity and their antioxidant activity, by a number of chemical assays. Assays employed included a β-carotene-linoleic acid (linoleate) model system, reducing power, scavenging effect on the DPPH• free radical and capacity to scavenge hydroxyl free radicals (HO•), by use of electron paramagnetic resonance (EPR) spectroscopy. The bearberry-leaf extract consistently exhibited the highest antioxidant activity based on the tests performed, and seems to be a promising source of natural antioxidants. The polyphenolic constituents appear to be responsible, at least in part, for the extract's radical-scavenging capacity. Research is progressing to characterize the antioxidant compounds in the bearberry-leaf extract and their mode of action in imparting antioxidant activity to various food systems.

Keywords: Plant extracts; Polyphenolics; Natural antioxidants; Free-radical scavenging capacity; Antioxidant activity; EPR spectroscopy

1. Introduction

Spices and herbs are added to food, not only for flavour, but also for preservation. Research has shown that constituents of these aromatic plants can function as natural antioxidants and thereby prevent/retard rancidity of food lipids, improve sensory scores and offer greater consumer acceptance of food products (Nakatani, 1997). Their efficacy depends highly upon the food system in question, their level of addition, and stability when subjected to food processing operations. Although certain culinary spices and herbs, or their fractions, possess marked antioxidant capacities, their practical application to foods may be restricted, due to a pungent and/or characteristic flavour imparted by the plant species.

A number of synthetic antioxidants, such as 2- and 3-*tert*-butyl-4-methoxyphenol (i.e. butylated hydroxyanisole, BHA), 2,6-di-*tert*-butyl-4-methylphenol (i.e. butylated hydroxytoluene, BHT) and *tert*-butylhydroquinone (TBHQ) have been added to foodstuffs but, because of toxicity issues, their use is being questioned (Valentão, Fernandes, Carvalho, Andrade, Seabra, & Bastos, 2002). Attention has therefore been directed toward the development/isolation of natural antioxidants from botanical sources, especially edible plants. The use of natural antioxidants in foods is limited, however, on account of the lack of knowledge concerning their molecular composition, the content of active compounds in the raw material and the availability of relevant toxicological data. Unlike synthetic antioxidants, which are phenolic compounds with varying degrees of alkyl substitution, natural antioxidants

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can be phenolic compounds (flavonoids, phenolic acids and tannins), nitrogen-containing compounds (alkaloids, chlorophyll derivatives, amino acids, peptides, and amines), carotenoids, tocopherols or ascorbic acid and its derivatives (Velioglu, Mazza, Gao, & Oomah, 1998). Crude extracts of spices, herbs and other plant materials rich in polyphenolics are increasingly of interest to the food industry because they have the capacity to retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary disease and cancer is also raising interest among scientists, food manufacturers and consumers since the future trend is toward functional foods with specific health effects (Löliger, 1991). The Canadian prairies have a wealth of untapped plant species which could be utilized, or constituents therefrom, in food systems as a source of natural antioxidants.

Reactive oxygenic species in the form of superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) are natural by-products of our body's metabolism. They are dangerous, however, when present in excess, and can attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with degenerative diseases (Jung, Park, Chung, Kim, & Choi, 1999; Pietta, Simonetti, & Mauri, 1998; Valentão et al., 2002). Although the mammalian body has certain defence mechanisms to combat and reduce oxidative damage, epidemiological evidence indicates that the consumption of foodstuffs containing antioxidant phytonutrients—notably flavonoids and other polyphenolics—is advantageous for our health (Cao, Booth, Sadowski, & Prior, 1998; Pulido, Bravo, & Saura-Calixto, 2000). There are frequent articles in newspapers or scientific literature citing the usefulness of the free-radical scavenging properties of antioxidants and their general benefits to human health. Flavonoids and other plant phenolics, such as phenolic acids, tannins, lignans and lignin, are especially common in the leaves, flowering tissues, and woody parts, such as stems, bark and roots, of plants. The antioxidant activity of these phenolics is mainly due to their redox properties, which allow them to act as reducing agents or hydrogen-atom donors. Thus, natural antioxidants function as free-radical scavengers and chain breakers, complexers of pro-oxidant metal ions and quenchers of singlet-oxygen formation (Pratt, 1992). Fortunately for the chemist, the unpaired electrons associated with free radicals are susceptible to detection by electron paramagnetic resonance (EPR) spectroscopy; however, the free radicals or secondary reaction products must survive long enough to permit detection. The formation of free radicals and their quenching by antioxidants can be monitored by EPR using spin-trapping techniques.

The purpose of this study is to evaluate six plant species, indigenous to the Canadian prairies, for their radical-scavenging properties and antioxidant activity, by use of a number of classical assays as well as by a not-so-common EPR procedure, and to assess whether these species could be sources of natural antioxidants for food applications.

2. Materials and methods

2.1. General

All solvents used were of ACS grade, unless otherwise specified. Ethanol [95% (v/v) technical grade] was purchased from Stanchem (Commercial Alcohols Division, Winnipeg, MB), while methanol and chloroform were acquired from the Fisher Scientific Company (Nepean, ON). The butylated hydroxyanisole (BHA), (+)-catechin, β-carotene, linoleic acid, monosodium phosphate, disodium phosphate, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), hydrogen peroxide, ferrous sulphate, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^\bullet$), Folin and Ciocalteu's phenol reagent and polyoxyethylenesorbitan monopalmitate (Tween 40) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Potassium ferricyanide, ferric chloride, sodium carbonate, Whatman No. 1 filter paper and trichloroacetic acid were purchased from VWR International (Mississauga, ON).

2.2. Herbs selected for investigation

A number of non-toxic plant species were selected for examination and acquired from the Department of Plant Sciences, University of Saskatchewan (Saskatoon, SK). All harvested plants had been dried and were stored in a herb room at 10 °C with a relative humidity of less than 50%, until used. The main criterion for herb selection was the content of phenolic constituents (i.e. phenolic acids and their esters, flavonoids, and other phenolic glycosides) within each herb. Particular attention was directed towards those plants with constituents containing free hydroxyl groups, as these classes of compound act as free-radical scavengers and possess antioxidant activity. A secondary criterion for herb selection was the herb's marketable potential as a specialized cash crop. In the present study, the plant species investigated included the roots of wild licorice (*Glycyrrhiza lepidota*), narrow-leaved echinacea (*Echinacea angustifolia*), senega (*Polygala senega*), leaves of bearberry (*Arctostaphylos uva-ursi*) and aerial parts of two varieties of horsetail (*Equisetum* spp.).

2.3. Preparation of herbal extracts

Plant parts were cut up and ground in a coffee mill (Moulinex Corporation, Toronto, ON). Prepared material

was transferred to dark-coloured flasks, mixed with 95% (v/v) ethanol at a material-to-solvent ratio of 15:100 (m/v) and placed in a shaking Magni Whirl constant-temperature bath (Blue M Electric Company, Model MSG-1122A-1, Blue Island, IL) at 50 °C for 30 min. Afterwards, the slurry was filtered through Whatman No. 1 filter paper and the residue was re-extracted twice more. Combined supernatants were evaporated to dryness under vacuum at 40 °C using a Büchi Rotavapor/Water bath (Models EL 131 and 461, respectively, Brinkmann Instruments [Canada] Ltd., Mississauga, ON). Prepared extracts were stored at 4 °C in air until further analysed.

2.4. Determination of total phenolics in the plant extracts

The total phenolics content in the plant extracts was estimated by a colorimetric assay based on procedures described by Swain and Hillis (1959) and Naczk and Shahidi (1989). Briefly, a 0.5-ml aliquot of plant extract dissolved in methanol was pipetted into a test tube containing 8 ml of distilled water. After mixing the contents, 0.5 ml of the Folin and Ciocalteu's phenol reagent and 1 ml of a saturated sodium carbonate solution were added. The contents were vortexed for 15 s and then left to stand at room temperature for 30 min. Absorbance measurements were recorded at 725 nm using a Milton Roy Spectronic Genesis 5 spectrophotometer (Fisher) and (+)-catechin was used in the construction of the standard curve. Estimation of the phenolic compounds was carried out in triplicate. The results are mean values and expressed as mg of (+)-catechin equivalents/g of extract.

2.5. Free-radical scavenging capacity and antioxidant activity assays

2.5.1. β-Carotene-linoleic acid (linoleate) assay

The antioxidative activity of crude extracts was evaluated using a β-carotene-linoleic acid (linoleate) model system (Miller, 1971). Briefly, 1 ml of β-carotene (0.2 mg/ml) dissolved in chloroform was pipetted into a small round-bottom flask. After removing the chloroform by using a rotary evaporator (Büchi), 20 mg of linoleic acid, 200 mg of Tween 40 and 50 ml of aerated distilled water were added to the flask with vigorous stirring. Aliquots (5 ml) of the prepared emulsion were transferred to a series of tubes containing 2 mg of extract or 0.5 mg of BHA. Each type of sample was prepared in triplicate. The test systems were placed in a water bath at 50 °C for 2 h. The absorbance of each sample was measured using a spectrophotometer (Milton Roy) set at 470 nm, immediately after sample preparation ($t = 0$ min) and at 15-min intervals until the end ($t = 120$ min) of the experiment.

Antioxidant activity was expressed in three different ways. First, the rate of β-carotene bleaching was calculated according to first-order kinetics, as described by Al-Saikhan, Howard, and Miller (1995). Thus:

$$\text{rate of } \beta\text{-carotene bleaching} = \ln(A_{t=0}/A_{t=t}) \times 1/t, \quad (1)$$

where $A_{t=0}$ is the initial absorbance (470 nm) of the emulsion at time 0; $A_{t=t}$ is the absorbance (470 nm) at 15, 30 and 45 min; and t is the time in min. Based on the rates determined at the 15, 30 and 45 min time intervals, an average rate was calculated. The antioxidant activity (ANT) was expressed as the percent inhibition of the rate of β-carotene bleaching relative to the aqueous control using the equation:

$$\%\ \text{ANT} = 100 \times \left(R_{\text{control}} - R_{\text{sample}}\right)/R_{\text{control}}, \quad (2)$$

where R_{control} and R_{sample} are the average bleaching rates of β-carotene in the emulsion without antioxidant and with plant extract, respectively. Second, the oxidation rate ratio (ORR) was calculated based on the equation described by Marinova, Yanishlieva, and Kostova (1994). Thus:

$$\text{ORR} = R_{\text{sample}}/R_{\text{control}}, \quad (3)$$

where R_{sample} and R_{control} are the same average rates as defined in Eq. (2). Third, the antioxidant activity was expressed as the percent inhibition of coupled oxidation of β-carotene and linoleic acid against the water and BHA control samples, based on absolute changes in absorbance measurements at two distinct points—60 and 120 min—during the assay rather than as an average rate. In Eq. (4) below, the results are normalized by invoking two extremes: (1) the water control which should offer no protection against oxidation of the linoleic acid/β-carotene emulsion; therefore, the antioxidant activity is defined as 0% for this system; and (2) the BHA control which should offer ca. 100% protection against oxidation over the time course of the assay. The antioxidant activity of the plant extracts under investigation was expressed as:

$$\%\text{AA} = 100 \times \left[1 - \left(A_{\text{E}}^{t=0} - A_{\text{E}}^{t=t}\right)/\left\{\left(A_{\text{W}}^{\text{t=0}} - A_{\text{W}}^{t=t}\right) + \left(A_{\text{BHA}}^{t=0} - A_{\text{BHA}}^{t=t}\right)\right\}\right] \quad (4)$$

where AA is the antioxidant activity, $A_{\text{E}}^{t=0}$ is the absorbance (470 nm) of the extract in question at 0 min, $A_{\text{E}}^{t=t}$ is the absorbance (470 nm) of the extract at $t = 60$ or 120 min, $A_{\text{W}}^{t=0}$ is the absorbance (470 nm) of the aqueous control sample at 0 min, $A_{\text{W}}^{t=t}$ is the absorbance (470 nm) of the aqueous control sample at $t = 60$ or 120 min, $A_{\text{BHA}}^{t=0}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at 0 min, and $A_{\text{BHA}}^{t=t}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at $t = 60$ or 120 min.

2.5.2. Reducing power assay

The reducing power of the prepared extracts was determined according to the method of Oyaizu (1986) and Yen and Chen (1995). Briefly, each extract (0.2–1.0 mg) was dissolved in 1.0 ml of distilled water to which was added 2.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50 °C for 20 min. Following this, 2.5 ml of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 1750×*g* for 10 min. A 2.5-ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm; increased absorbance of the reaction mixture indicates greater reducing power. Mean values from three independent samples were calculated for each extract.

2.5.3. Radical-scavenging activity (RSA) assay

The capacity of prepared extracts to scavenge the 'stable' free radical 2,2-diphenyl-1-picrylhydrazyl• (DPPH•) was monitored according to the method of Hatano, Kagawa, Yasuhara, and Okuda (1988), with some slight modifications. Extracts (0.025–2.000 mg) were dissolved in 4 mL of methanol and then added to a methanolic solution of DPPH• (1 mM, 0.5 ml). The mixture was vortexed for 15 s and then left to stand at room temperature for 30 min. The absorbance of the resulting solution was read spectrophotometrically at 517 nm. A methanolic solution of DPPH• that had decayed and hence no longer exhibited a purple colour (i.e. 2 mg of BHA dissolved in 4 ml of methanol with 0.5 ml of the DPPH• solution added) was chosen for background correction, instead of pure methanol. The radical scavenging activity (RSA) was calculated as a percentage of DPPH• discolouration using the equation:

$$\% \quad \mathrm{RSA} = 100 \times (1 - A_E/A_D), \tag{5}$$

where A_E is the absorbance of the solution when an extract has been added at a particular level, and A_D is the absorbance of the DPPH• solution with nothing added.

2.5.4. Electron paramagnetic resonance (EPR) spectroscopy investigations

To generate the hydroxyl radical (HO•) so as to test the antioxidant efficacy of the prepared plant extracts, the Fenton (Haber–Weiss) reaction was used. Ferrous sulphate reacts with hydrogen peroxide in the following manner:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \tag{6}$$

The generated HO• radical reacts rapidly with either the added antioxidant or the nitrogenic spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO); the resultant DMPO-HO• radical adduct is a stable spin trap that is detectable by EPR spectroscopy (Harbour, Chow, & Bolton, 1974). DMPO and H_2O_2 were prepared in a 0.1 M phosphate buffer, pH 7.2, while ferrous sulphate and plant extracts were dissolved in distilled water (Shi, Dalal, & Jain, 1991). The control reaction mixture consisted of 20 μl each of 0.3 M DMPO, 10 mM H_2O_2, 10 mM ferrous sulphate and water, so as to give final concentrations of 0.075 M DMPO, 2.5 mM H_2O_2 and 2.5 mM ferrous sulphate. For test samples, the water was replaced by 20 μl of extract solutions containing 0.5, 1.0, 2.0 and 4.0-mg quantities. Half of the test mixture was transferred to a capillary tube: the capillary containing the sample in air was sealed with a silicone sealant (Dow Corning high vacuum grease), put into an EPR quartz tube (159 mm length, 3.00 mm o.d., 2.04 mm i.d. Wilmad Glass, Buena, NJ), and then scanned by the EPR spectrometer exactly 3 min after ferrous sulphate had been added to the sample. The EPR scan was completed 14 min later.

The EPR measurements of the free radicals were performed using an X-band continuous-wave ($\nu = 9.4$ GHz) Bruker ESP-300 spectrometer equipped with a Bruker ER-4107 WZ high-sensitivity resonator (Bruker-Biospin, Karlsruhe, Germany). Operating parameters for the EPR spectrometer were as follows: microwave power 10 mW, centre field 3340 G, sweep width 70.0 G, conversion time 163.84 ms, time constant 81.92 ms, modulation frequency 50 kHz, modulation amplitude usually 1.0 G, receiver gain 1×10^5, number of scans per sample 5, and a temperature of 22±1 °C. All scans were recorded using the same instrument settings and sample position, and were carried out in diffuse room light. For any given type of experiment, three sample preparations were recorded.

The magnetic-field values of all experimental EPR spectra were adjusted by measuring a sample of 0.1 mM DPPH• in benzene under identical conditions to those for the spin-adduct samples. The magnetic field was corrected according to the known *g* value of 2.00354 for DPPH• (Weil, Bolton, & Wertz, 1994). The difference between fields at the location of the proton NMR gaussmeter and the sample of interest was calculated. Thus the position of each EPR line obtained from the samples analysed was adjusted by adding 0.95±0.07 G. To simulate the experimental EPR spectrum, the computer programmes WinSim and EPR-NMR were used (Duling, 1994; Mombourquette & Weil, 2000). Each simulated spectrum consists of first-derivative intensity values plotted against 4096 points of magnitude of the corrected applied magnetic field. The relevant spectroscopic parameters, namely the *g* value, hyperfine coupling constants and linewidths, were employed in the production of a plot so that correct identification of the free radicals, which often show only slight differences in the spin-hamiltonian parameters, could be determined.

The relative intensity of the spin adducts was assessed by calculating the area under the quartet EPR absorption spectrum formed by each sample (i.e. in the presence of the extract) and then comparing this number to that of the control system (i.e. in the absence of the extract).

2.6. Statistical analysis

Each extract, for the purpose of statistical analysis, was considered as a "treatment." All measurements were replicated three times for each treatment and their means are reported, unless otherwise specified. Linear regressions between the content of total phenolics and data for some of the antioxidant assays were assessed.

3. Results and discussion

3.1. General

After scrutinizing some background information from the Saskatchewan herb data base pertaining to various wild- and cultivated-plant species found in the province and a few others grown on the prairies (Barl, Loewen, & Svendsen, 1996), six herbs were selected for examination: the roots of wild licorice (*Glycyrrhiza lepidota*), narrow-leaved echinacea (*Echinacea angustifolia*), senega (*Polygala senega*), the leaves of bearberry (*Arctostaphylos uva-ursi*) and the aerial parts of two varieties of horsetail (*Equisetum* spp.).

The contents of total phenolic compounds in crude ethanolic extracts obtained from each plant species are presented in Table 1; the results are reported as mg (+)-catechin equivalents/g of extract. As is clearly evident, the content of polyphenolic compounds ranged quite markedly, from a low of 58 mg/g for one of the horsetail (1994) samples to a high of 312 mg/g for the bearberry-leaf extract. The yield of the crude ethanolic extract from the bearberry sample was second greatest at ca. 27% of all extracts collected, but it had the highest level of polyphenolics (Amarowicz, Barl, & Pegg, 1999). Although the extract from the horsetail (1996) sample contained the second largest percentage of total phenolics, it had a very low yield of only 3%. On the other hand, the ethanolic extract from senega root afforded the greatest yield (ca. 33%), but its total phenolic content was only 23% of that found in the bearberry-leaf extract.

3.2. Free-radical scavenging capacity and antioxidant activity assays

3.2.1. β-Carotene-linoleic acid (linoleate) assay

Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was employed as an antioxidant test reaction (see Fig. 1). In this particular model, β-carotene undergoes rapid discolouration in the absence of an antioxidant. During oxidation, an atom of hydrogen is abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds (Frankel, 1998). The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lose their conjugation, the carotenoids lose their characteristic orange colour. Fortunately, this process can be monitored spectrophotometrically. The presence of a phenolic antioxidant can hinder the extent of β-carotene destruction by "neutralizing" the linoleate free radical (i.e. utilizing its redox potential) and any other free radicals formed within the system. Hence, this forms the basis by which plant extracts can be screened for their antioxidant potential.

Antioxidative activity was observed in the crude ethanolic extracts of all six plant species screened (Table 1). In this work, the greatest antioxidative efficacy found was from the synthetic antioxidant (i.e. BHA) control, which practically inhibited β-carotene consumption throughout the incubation period. When evaluating the bleaching rates of β-carotene (i.e. %

Table 1
Antioxidant activity and total phenolics of ethanolic plant extracts[a]

Crude ethanolic extract of various plant species[b]	ANT (%)	ORR	AA $t=60$ min (%)	AA $t=120$ min (%)	Total phenolics[c] (mg/g)
Horsetail (1996), aerial parts (*Equisetum* spp.)	69.1	0.309	51.8	35.7	216
Horsetail (1994), aerial parts (*Equisetum* spp.)	60.0	0.420	39.0	20.5	58
Bearberry leaves (*Arctostaphylos uva-ursi*)	98.2	0.018	96.7	95.3	312
Narrow-leaved echinacea root (*Echinacea angustifolia*)	64.8	0.352	63.4	58.0	62
Senega root (*Polygala senega*)	70.8	0.255	70.7	66.8	72
Wild-licorice root (*Glycyrrhiza lepidota*)	74.5	0.255	76.8	75.6	63

[a] ANT, antioxidant activity based on average rate of β-carotene bleaching; ORR, oxidation rate ratio; AA, antioxidant activity of extract at $t=60$ and 120 min. For complete definitions of the relevant equations, refer to the "Materials and methods" section.

[b] Taxonomical classifications of plants are by their genus and species. In some cases the exact species is not known and the abbreviation spp. is used to denote all possible species.

[c] Results for total phenolics are expressed as mg of (+)-catechin equivalents/g of extract.

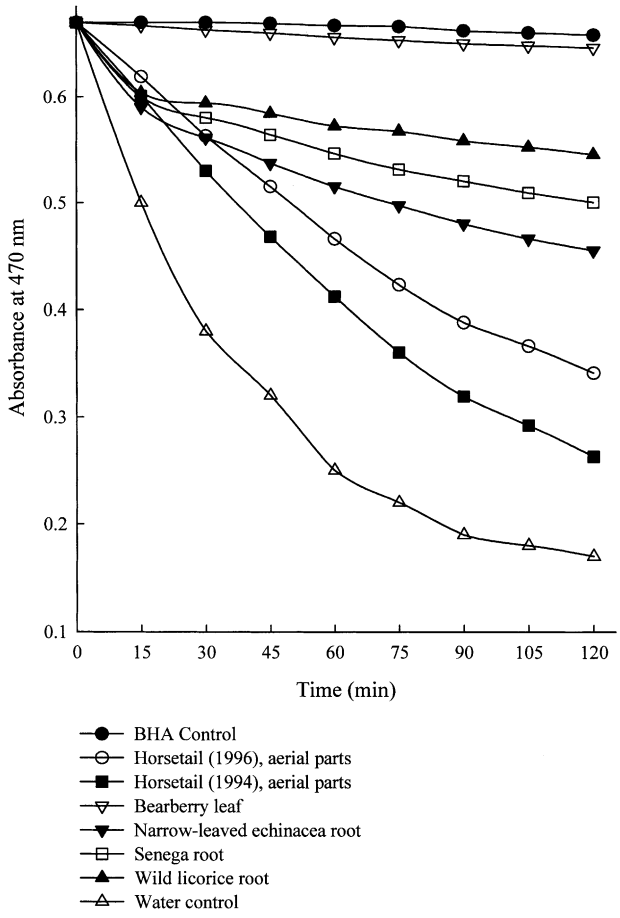

Fig. 1. Antioxidative activity of ethanolic extracts from plant species, as assessed by the coupled oxidation of β-carotene and linoleic acid over 120 min. Symbols used for the BHA control, water control and extracts from horsetail (1996) aerial parts, horsetail (1994) aerial parts, bearberry leaves, narrow-leaved echinacea root, senega root and wild-licorice root are described within the figure.

ANT—Table 1), the antioxidant activity of plant extracts ranged from 60.0 to 98.2% for that of the horsetail (1994) and bearberry-leaf preparations, respectively. The marked activity of the bearberry-leaf extract is partially attributed to its higher content of polyphenolics (cf. 58 mg (+)-catechin equivalents/g of extract in horsetail (1994) to 312 mg/g extract in bearberry). Although the bearberry-leaf extract possessed the highest % ANT and content of total phenolics and the horsetail (1994) extract exhibited the lowest % ANT and content of total phenolics, the % ANT of the horsetail (1996) extract was only 69.1%. This preparation contained 216 mg (+)-catechin equivalents/g of extract; nevertheless, its antioxidant activity was lower than those determined for senega root and wild-licorice root extracts, which contained 72 and 63 mg (+)-catechin equivalents/g of extract, respectively. The low antioxidant activity attributed to the horsetail (1996) extract was somewhat surprising considering the amount of total phenolics it contained. Regression analysis between the antioxidant activity and the content of

Table 2
Effect of plant extracts on the EPR signal intensity of the DMPO-HO• spin adduct[a]

Crude ethanolic extract of various plant species present at different parts-per-million (ppm) quantities[b]	% Hydroxyl radical (HO•) scavenging capacity[c]
Horsetail (1996), aerial parts (Equisetum *spp.*)	
250	39.5
500	42.2
1250	71.1
2500	86.8
Horsetail (1994), aerial parts (Equisetum *spp.*)	
250	18.3
500	26.0
1250	49.3
2500	72.3
Bearberry leaves (Arctostaphylos uva-ursi)	
12.5	53.4
25	94.7
50	98.8
125	100
250	100
Narrow-leaved echinacea root (Echinacea angustifolia)	
250	35.2
500	72.2
1250	78.8
2500	100
Senega root (Polygala senega)	
250	31.6
500	37.0
1250	61.9
2500	100
Wild-licorice root (Glycyrrhiza lepidota)	
250	30.8
500	38.0
1250	77.3
2500	100
Green tea (Camellia *spp.*)	
12.5	14.1
25	65.7
50	80.2
125	100
250	100

[a] Taxonomical classifications of plants are by their genus and species. In some cases the exact species is not known, so the abbreviation spp. is used to denote all possible species.

[b] Instead of specifying the mass (in mg quantities) of plant extract added to the EPR tube, ppm addition levels are indicated. For example, a 250-ppm addition means that a 20 μl aliquot of an extract solution at a concentration of 1 mg/ml was added to the assay medium (total volume was 80 μl). The density of the DMPO, H_2O_2, $FeSO_4$ and plant extract mixture was assumed to be ~1.0 g/ml; hence, the extract is present at a 250-ppm concentration.

[c] The values represent the % suppression of EPR signal for the DMPO-HO• adduct when an extract is present in the medium, at various levels.

total phenolics, for all six plant extracts, was not significant ($P > 0.05$). Correlation coefficients (r^2), based on linear regressions between the antioxidant activity data calculated by the three different methods in this study and the content of total phenolics, ranged from 0.641 to 0.161. Velioglu et al. (1998) examined 28 plant products and found significant relationships between the total phenolics and antioxidant activity for flaxseed and cereal products, but not for the anthocyanin-rich materials and medicinal plants. These authors suggested that, besides the composition of the phenolics, other factors can play a major role in the antioxidant activity of plant materials.

The results from % ANT are mirrored by the ORR data, where a lower ORR denotes better antioxidant activity by the extract in question. A similar trend was observed in the antioxidant activity results at the 60 and 120-min points during the assay, except for the horsetail (1996) sample: its antioxidant potential was less than that of the ethanolic extract from narrow-leaved echinacea roots. The normalized antioxidant data at 60 and 120-min of incubation probably reflect the antioxidant activity of the extracts more accurately than either of the other two definitions cited above. The relative inhibitions of β-carotene consumption, after 60 min of incubation, by the ethanolic extracts of bearberry leaf, licorice root and senega root were ca. 97, 77 and 71%, respectively, based on Eq. (4). After a further incubation period of 60 min, the inhibition of β-carotene consumption for these extracts were ca. 95, 76 and 67%, respectively. Minimal activity against β-carotene consumption was noted for extracts from the 1996 and 1994 horsetail species; after 120 min of heating, the % activities observed were only 36 and 21%, respectively.

Although it is believed that the total number of hydroxyl groups present in the aromatic constituents of an extract, in part, offers better antioxidative properties, compounds present in ethanolic extracts belong to different classes of phenolics. These classes most likely have varying antioxidative strengths and that synergism of polyphenolics, with one another and/or other components present in an extract may contribute to the overall observed antioxidant activity (Shahidi, Wanasundara, & Amarowicz, 1994). Kähkönen et al. (1999) reported that no significant correlations could be found between the total phenolic content and antioxidant activity of 92 plant extracts in any of the studied subgroups. These authors commented that different phenolic compounds show different colorimetric responses when using the Folin–Ciocalteu reagent. Similarly the molecular antioxidant response of phenolic compounds to a lipid substrate varies remarkably, depending on chemical structure and the oxidation conditions. Thus, the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content. The molecular structures of phenolics play a vital role in their antioxidant activity. For example, Pokorný (1987) reported that substituted monophenolic compounds exhibited a greater antioxidant activity than unsubstituted monophenolics. In the case of bearberry, its antioxidative activity in the β-carotene-linoleate model system was more efficacious than that observed for extracts from other plant sources (Amarowicz & Shahidi, 1995; Amarowicz, Karamać, Wanasundara, & Shahidi, 1997; Amarowicz, Karamać, Kmita-Głażewska, Troszyńska, & Kozłowska, 1996; Amarowicz, Troszyńska, Karamać, & Kozłowska, 1996; Amarowicz, Wanasundara, Karamać, & Shahidi, 1996; Amarowicz, Piskuła, Honke, Rudnicka, Troszyńska, & Kozłowska, 1995; Amarowicz, Wanasundara, Wanasundara, & Shahidi, 1993; Chavan, Amarowicz, & Shahidi, 1999; Wanasundara, Amarowicz, & Shahidi, 1994; Wanasundara, Amarowicz, & Shahidi, 1996; Żegarska, Rafałowski, Amarowicz, Karamać, & Shahidi, 1998), and its polyphenolic content is partially responsible for this observation.

3.2.2. Reducing power assay

Fig. 2 depicts the reducing powers of the crude ethanolic extracts of the six plant species examined as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each extract. The presence of reductants (i.e. antioxidants) in the herbal extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung, Chang, Chao, Lin, & Chou, 2002). In other words, the $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the "semi-quantitative" determination of dilute concentrations of polyphenolics, which participate in the redox reaction. At a 1-mg dose, the reducing power of bearberry-leaf extract was far superior to that of any other extract investigated. In fact, the bearberry-leaf extract was ca. 2.5 times superior to that of the second most powerful extract, the horsetail (1996) sample, and possessed ca. a 13 times greater redox potential than the senega root extract. The reducing powers of the remaining extracts were quantitatively similar to one another, and quite low. Polyphenolics in the bearberry-leaf extract appear to function as good electron and hydrogen-atom donors and therefore should be able to terminate radical chain reactions by converting free radicals to more stable products. Furthermore, the reducing power of the bearberry-leaf extract (i.e. 2.26 absorbance units @ 1-mg dose) was of the same magnitude as that of a green-tea extract (results not shown). Similarly, Yen and Chen (1995) reported that the reducing powers of green, pouchong, oolong and black-tea extracts were 2.47, 2.38, 2.75 and 1.32 at a 1-mg dose, respectively. Another interesting observation was that

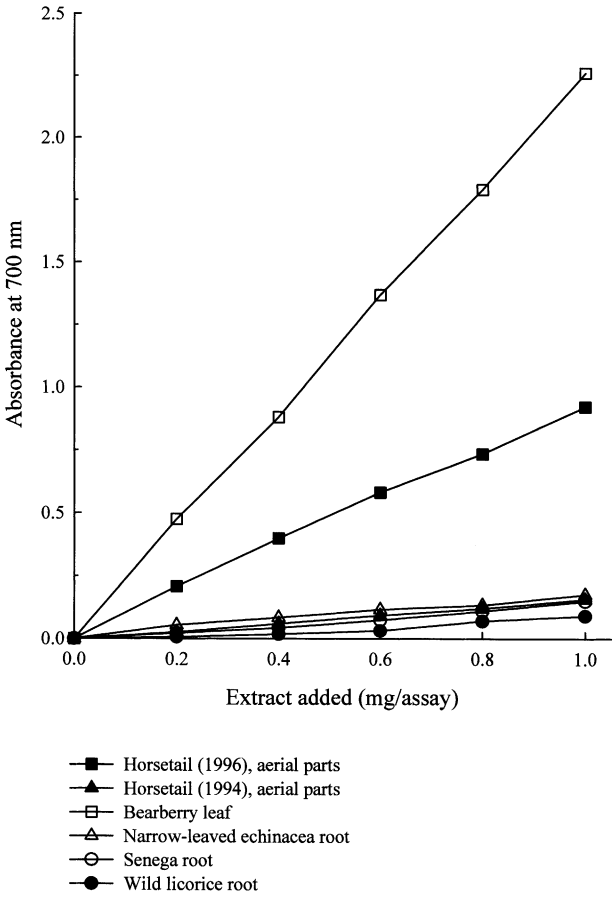

Fig. 2. Reducing powers of ethanolic extracts from selected Saskatchewan plant species. Symbols used for the extracts from horsetail (1996) aerial parts, horsetail (1994) aerial parts, bearberry leaves, narrow-leaved echinacea root, senega root and wild-licorice root are described within the figure.

the data, for all six plant species from this assay, correlated well with the content of total phenolics. Using dose concentrations from the assay of 0.4 mg and 1 mg as examples, the linear correlation coefficients (r^2, $P<0.05$) between the observed reducing power and content of total phenolics were 0.945 and 0.961, respectively. The results from these two assays do not entirely mirror the data from the β-carotene-linoleate test, in which the crude extracts from the wild-licorice, senega and narrow-leaved echinacea roots were better at quenching β-carotene consumption than the horsetail (1996) sample.

3.2.3. Radical-scavenging activity (RSA) assay

The RSA of the prepared plant extracts was tested using a methanolic solution of the 'stable' free radical, DPPH•. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH• has the advantage of being unaffected by certain side reactions, such as metal-ion chelation and enzyme inhibition, brought about by various additives. A freshly prepared DPPH• solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH• free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH• molecule) and convert them to a colourless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at the 517 nm band (Yamaguchi, Takamura, Matoba, & Terao, 1998). Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract in terms of hydrogen atom-donating capacity. Caution must be exercised, however, when interpreting such results. The reactions that DPPH• can undergo are not simple and straightforward, and much is still to be learned about its chemistry. One cannot arbitrarily assume that the decrease in absorbance at the 517 nm absorption maximum is solely attributed to the antioxidant donating a hydrogen atom or an electron to DPPH•. Nevertheless, the "DPPH• test" is a commonly employed assay in antioxidant studies and offers a rapid technique in which to screen the RSA of pure synthetic compounds, isolated natural compounds, crude plant extracts and foods. It is also important to note that the DPPH• test only recognizes free-radical scavenging effects and not pro-oxidant activity. Certain additives can function as both antioxidants and pro-oxidants; the food product or system in question and the concentration at which they are employed dictate their action. Brand-Williams, Cuvelier, and Berset (1995) modified the DPPH• test to account for differences in the kinetic behaviours of the added antioxidants. Employing this approach is limited, however, to the evaluation of the radical-scavenging potential of pure antioxidants with known molecular structure.

The RSA values of the crude ethanolic extracts from the six plant species were examined and compared against one another. Fig. 3 depicts the dose–response curves for the RSA of the plant extracts; results are expressed as a percentage of the ratio of the decrease in absorbance at 517 nm to the absorbance of DPPH• solution in the absence of phenolics at 517 nm (Yoshida et al., 1989). At a dosage of 1 mg, the ethanolic extract from bearberry leaf afforded greater than 99.5% RSA on the stable DPPH• free radical, followed by horsetail (1996), wild-licorice root, senega root and narrow-leaved echinacea root at 93.9, 49.7, 48.5 and 36.1%, respectively. The extract from the aerial parts of horsetail (1994), which contained the lowest amount of polyphenolics, exhibited the weakest RSA values of 12.8 and 23.7% at 1 and 2-mg levels of addition, respectively. The data were in agreement with those measured from the reducing power assay, which is to be expected. The RSA of bearberry-leaf extract was far superior to any of the other extracts investigated, as well as green and black-tea

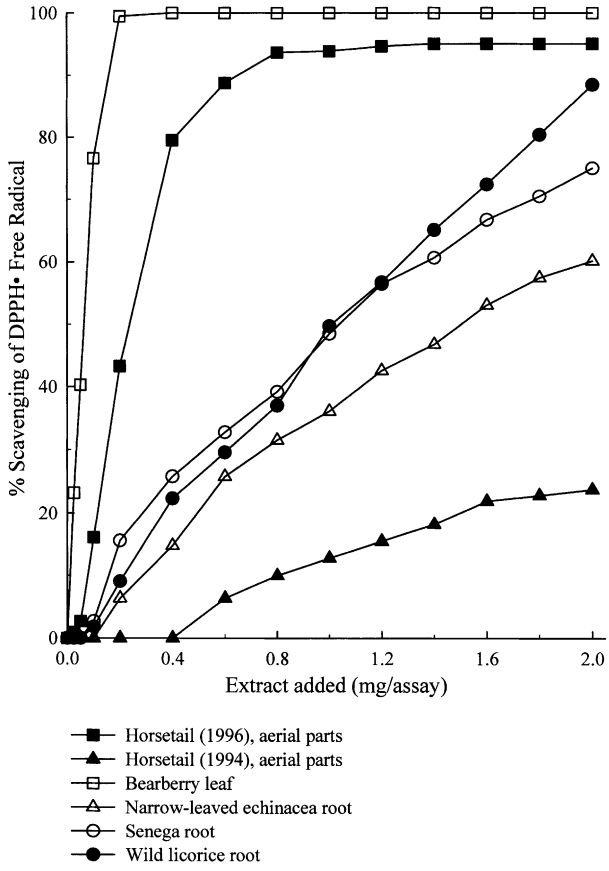

Fig. 3. Scavenging effect of plant extracts on 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH•). Symbols used for the extracts from horsetail (1996) aerial parts, horsetail (1994) aerial parts, bearberry leaves, narrow-leaved echinacea root, senega root and wild-licorice root are described within the figure.

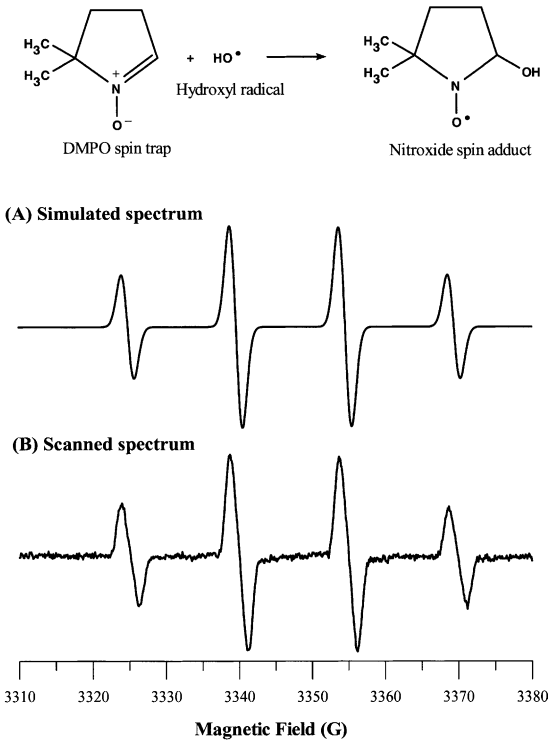

Fig. 4. Simulation of the X-band EPR spectrum of free radicals formed by the reaction of aqueous 0.075 M DMPO, 2.5 mM H_2O_2 and 2.5 mM ferrous sulphate in air at room temperature.

extracts reported by Yen and Chen (1995); these authors stated that the scavenging effects of tea extracts ranged from 49 to 66% at a 2-mg level of extract. In the present study, RSA values of 40.3 and 76.6% were observed for the bearberry-leaf extract at 0.05 and 0.1-mg levels of addition; however, a direct comparison of the data cannot be made because, in the assay used by Yen and Chen (1995), the volume of the DPPH• solution was double. It could be fruitful to study the RSA of the bearberry-leaf extract on an equimolar basis with the DPPH• free radical to help assess whether the extract is acting as a primary antioxidant; however, this first requires specific knowledge of the active components within the extract (e.g. if polyphenolics, how many phenolic hydroxyl groups are able to donate hydrogen atoms and are these groups well positioned so that a good electron delocalization and stabilization of the formed phenoxyl radicals result?), their respective concentrations and whether or not they exert a synergistic effect with each other.

Radical scavengers may protect cell tissues from free radicals, thereby preventing diseases such as cancer (Nakayama, Yamada, Osawa, & Kawakishi, 1993). Even though it is unclear whether active constituents in plant extracts, such as those from bearberry leaf, are active against free radicals after being absorbed and metabolised by cells in the body, radical-scavenging assays have gained acceptance for their capacity to rapidly screen materials of interest.

3.2.4. Electron paramagnetic resonance (EPR) spectroscopy investigations

Spin trapping is an EPR technique in which, typically, a transient free radical reacts with a diamagnetic molecule (i.e. the spin trap) via an addition reaction to form a stable free-radical product (i.e. the spin adduct). The spin adduct so formed is paramagnetic and exhibits an EPR spectrum with a hyperfine splitting parameter and *g*-value characteristic of the type of reactive free radical trapped. The reaction of Fe^{2+} with hydrogen peroxide (i.e. the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^-$) in the presence of the nitrone spin-trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), generates a spin adduct with an EPR signal, that is a 1:2:2:1 quartet with hyperfine coupling parameters $a(^{14}N) = a(^{1}H) = 14.9$ G (Buettner, 1987). Fig. 4 depicts the spectrum of the hydroxyl radical adduct with DMPO, showing this quartet signal (Scanned spectrum). EPR spectra obtained with the Fenton reaction system were simulated using the programmes WinSim and EPR-NMR (Duling, 1994; Mombourquette & Weil, 2000) and gave the fol-

lowing parameters: $g = 2.0058 \pm 0.0003$, $a(^{14}N) = 14.98 \pm 0.09$ G, $a(^{1}H) = 14.95 \pm 0.07$ G, lineshape = gaussian, and linewidth = 1.70 ± 0.03 G. These values are consistent with those found in the literature.

DMPO is the most widely used spin trap for the study of oxygen-centred free radicals such as the hydroxyl radical, HO•. The nitrone spin trap reacts via a carbon located in a beta position relative to the nitroxyl functional group (Fig. 4). The effects of natural product extracts possessing antioxidative activity on the EPR signal intensity of the DMPO-HO• adduct can be monitored and are expected to show a dose-dependent inhibition of the signal. There are several potential complications, i.e. means by which antioxidative compounds can influence the observed EPR signal. They can: (1) donate a hydrogen atom or an electron to the free-radical spin adduct and generate a non-radical product, thereby resulting in a decrease/loss in the intensity of the observed EPR spectrum; (2) compete with the diamagnetic spin trap at scavenging hydroxyl radicals and therefore cause a decrease in the intensity of the observed EPR spectrum for DMPO-HO•, and possibly give rise to a new EPR signal resulting from a resonance-stabilized antioxidant radical (NB, even if an antioxidant radical forms, it may not be observed because the EPR spectrometer has been tuned to pick up the stable DMPO-HO• radical signal); (3) react with one or both of the reactants in the Fenton reaction (i.e. any depletion of H_2O_2 or Fe^{2+} will suppress the generation of the hydroxyl radical); and (4) have an effect on the spin trap itself, so as to reduce its capacity to trap a free radical. A number of control runs were carried out when designing the experiment to show that the EPR spectrum did indeed result from DMPO trapping hydroxyl radicals generated by the Fenton reaction. Experiments are also necessary to determine whether the plant extracts have the capacity to scavenge HO• or to impede DMPO-HO• adduct formation by interfering with the mechanism of HO• formation or the capacity of the spin trap to react with them. Since the active constituents in the extracts tested are unknown, the mechanism(s) by which the extract is suppressing the EPR signal generated from the DMPO-HO• radical cannot be verified. Nevertheless, a hypothesis can be proposed for extracts demonstrating-radical scavenging potential.

In the present study, ethanolic extracts from all six plant species were examined by EPR spectroscopy for their capacity to act as free-radical acceptors and to limit DMPO-HO• adduct formation. All extract samples were tested in parallel with a control that did not contain any plant extract. Exactly 3 min after mixing DMPO, H_2O_2 and ferrous sulphate in air at room temperature, the sample showed an EPR signal exhibiting the 1:2:2:1 quartet pattern. To quantify the relative amount of spin trap present in a given sample, double integration of each EPR signal was performed. Table 1 shows the concentration-dependent degree of protection in which the plant extracts suppressed the EPR signal for the DMPO-HO• adduct at pH 7.2. All prepared extracts were able to suppress the observed EPR signal from the DMPO-HO· adduct, but to varying degrees. Our results are in concordance with those of other authors who have shown that polyphenolics from plants or their fractions have direct scavenging activities against hydroxyl radicals (Yen & Duh, 1994; Wettasinghe & Shahidi, 2000; Matthäus, 2002).

The extract from bearberry leaf was most prominent in its capacity to act as a free-radical acceptor and to limit DMPO-HO• formation. The characteristic 1:2:2:1 quartet signal for the DMPO-HO• adduct is barely detectable in some EPR spectra for systems containing the bearberry-leaf extract (Fig. 5); only when a 50-ppm concentration of the leaf extract, or less, was present in the assay medium was the splitting pattern of the DMPO-HO• signal evident. At higher levels of addition, no signal was detected, thereby indicating the concentration-dependent manner of the extract at reducing the intensity of the EPR signal for the DMPO-HO• radical. There are two likely possibilities for the efficacy of the bearberry-leaf extract: (1) the polyphenolic constituents of the bearberry-leaf extract are competing—albeit more effectively—with the diamagnetic spin trap at scavenging hydroxyl radicals and therefore resulting in

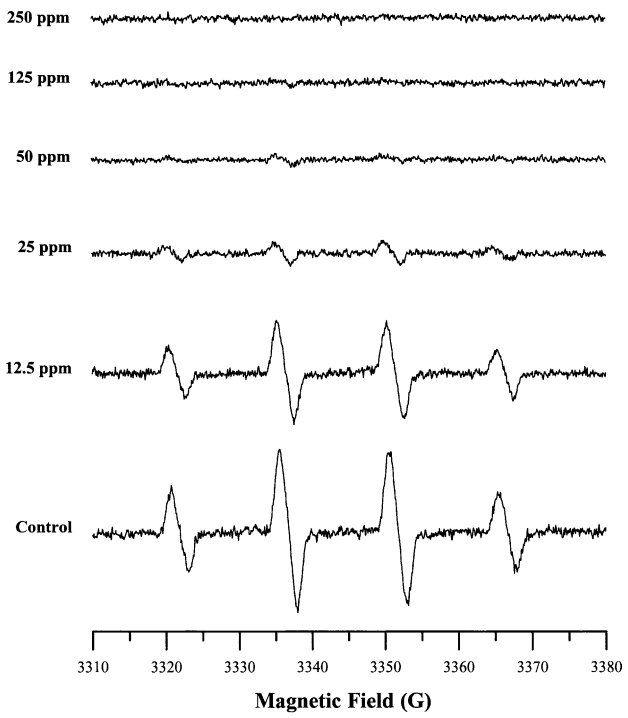

Fig. 5. X-band electron paramagnetic resonance spectra showing the effect of bearberry-leaf extract on the scavenging of hydroxyl radical (HO•) and retardation of DMPO-HO• adduct formation, in aqueous solution at room temperature in air.

a decrease in the intensity of the observed EPR spectrum for the DMPO-HO• radical; (2) the polyphenolics may inhibit the generation of the hydroxyl radical from the Fenton reaction due to chelation of iron(II) by constituents of bearberry leaf, the likely candidates being the tannins. It is also possible that both of these suggested mechanisms are working in tandem and are responsible for the suppression of the EPR signal from DMPO-HO•. If the latter hypothesis of the two is accurate, then the bearberry leaf is not functioning as a dioxygen scavenger; rather, it is behaving as a metal-ion chelator. The fact that the bearberry-leaf extract functions as an effective antioxidant, in the aforementioned assays, tends to support the notion that some constituents within the crude extract can function as hydrogen-atom donors and oxygen scavengers.

In comparison with the efficacy of the bearberry-leaf extract at limiting DMPO-HO• adduct formation, both horsetail extracts were very poor candidates. Even at higher addition levels, the horsetail samples could not significantly reduce the signal intensity (data not shown), thereby indicating their poor efficacy as a source of natural antioxidants. The scavenging capacity of the bearberry-leaf extract was comparable to that of an ethanol extract from green tea, whose content of total phenolics was 479 mg (+)-catechin equivalents/g of extract. Green-tea leaves (Shanghai Tea Import & Export Corporation, Product of the People's Republic of China) were procured from a local merchant and an ethanolic extract was prepared from them in an identical manner to the herb extracts under investigation. Catechins are the major polyphenols in green tea and belong to the family of flavonoids; tea catechins have been shown to possess strong scavenging activity against superoxide anion, singlet oxygen and hydroperoxy radicals. They have a variety of physiological functions and can act as antioxidants in vitro and in vivo (Nanjo, Goto, Seto, Suzuki, Sakai, & Hara, 1996). Yen and Chen (1995) reported that tea extracts can scavenge hydroxyl radicals and consequently suppress the signal intensity of the DMPO-HO• free-radical adduct. In their study, the green-tea extract suppressed the EPR signal by 77.0, 91.9 and 100% when added to the system at concentrations of 250, 500 and 1000 ppm, respectively. The fact that the bearberry-leaf extract was more efficacious at limiting the EPR signal from the DMPO-HO• adduct than the green-tea extract in both Yen and Chen's and our own investigations, suggests that it is could be a very promising source of natural antioxidants.

4. Conclusions

In this study, the ethanolic extracts of six plant species from the Canadian prairies were found to possess radical-scavenging and antioxidant activities, as determined by a β-carotene-linoleic acid model system, reducing power, scavenging effect on the DPPH• free radical and scavenging capacity of hydroxyl free radicals (HO•) by electron paramagnetic resonance spectroscopy tests, but to varying degrees. The results gained from these assays provide simple data that make it possible to classify extracts with respect to their antioxidant potential. Because antioxidant activity does not always correlate with presence of large quantities of polyphenolics, the phenolic content and antioxidant activity data need to be examined, together, when screening plant extracts. In this investigation, the bearberry-leaf extract was found to be quite active and its potency, as illustrated by the EPR assay, was of the same magnitude as that of a green-tea extract. Research is progressing to characterize the antioxidant compounds in the bearberry-leaf extract as well as their mode of action in imparting antioxidant activity to different food systems.

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