ELSEVIER

Food Chemistry 70 (2000) 17–26

Food Chemistry

www.elsevier.com/locate/foodchem

Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals

Mahinda Wettasinghe, Fereidoon Shahidi *

Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, Canada, A1B 3X9

Received 28 April 1999; received in revised form 16 September 1999; accepted 18 September 1999

Abstract

Crude extracts of meals of borage and evening primrose were prepared under optimum extraction conditions and were subjected to Sephadex LH-20 column chromatography. Six fractions from each of the crude extracts were obtained and their content of total, hydrophilic and hydrophobic phenolics determined. The crude extracts and their fractions [at 100 and 200 ppm as sinapic acid (for borage) or catechin (for evening primrose) equivalents] were investigated for their reactive-oxygen species- (ROS; H_2O_2, $O_2^{\bullet-}$, $^{\bullet}OH$) and 2,2-diphenyl-1-picrylhydrazyl- ($DPPH^{\bullet}$) scavenging efficacies. Both types of crude extracts and their fractions exerted a concentration-dependent scavenging of ROS and $DPPH^{\bullet}$. At 200 ppm, borage crude extract and its fractions III, IV and V exhibited a 100% scavenging of H_2O_2 whereas evening primrose crude extract and its fraction III, at the same concentration, scavenged H_2O_2 completely. A complete quenching of $O_2^{\bullet-}$ was evident for assay media containing 200 ppm borage and evening primrose crude extracts/fractions with the exception of borage fraction V and evening primrose fraction I which showed about 75% quenching. At 200 ppm, borage and evening primrose crude extracts/fractions (except borage fractions I and III) exerted a complete quenching of $^{\bullet}OH$. Among the borage and evening primrose crude extracts/fractions investigated, only fraction VI of evening primrose, at 200 ppm, was able to completely quench $DPPH^{\bullet}$.

1. Introduction

Free radicals can be defined as species with an unpaired electron. The reactivity of free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high, as in the case of the short-lived and highly reactive hydroxyl radical ($^{\bullet}OH$) (Packer, 1994; Packer & Glazer, 1990). Fatty acids are susceptible to attack by highly reactive oxygen species (ROS) such as $^{\bullet}OH$; hence any reaction or process which forms ROS would definitely stimulate lipid oxidation. Hydrogen abstraction is easier in unsaturated fatty acids than in their saturated counterparts, thus making them more susceptible to ROS attack.

Oxygen and ROS are among the major sources of primary catalysts that initiate oxidation in vivo and in vitro. The electronic structure of oxygen has two unpaired electrons at energy levels of p antibonding, in triplet state ($^3\Sigma_g$; Korycka-Dhal & Richardson, 1978). The reaction of oxygen with ground state molecules of singlet multiplicity [i.e. polyunsaturated fatty acids (PUFA)] is spin-forbidden. However, this barrier does not apply to reactions which involve single electrons, hydrogen atoms, and molecules containing unpaired electrons, such as transition metal complexes and free radicals. Therefore, the triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) (Borg, 1993; Halliwell & Gutteridge, 1985; Kanner, German & Kinsella, 1987; Packer & Glazer, 1990).

Superoxide radical ($O_2^{\bullet-}$) is generated by four electron reduction of molecular oxygen into water. This radical is also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide radical ($O_2^{\bullet-}$) is also formed by activated phagocytes (monocytes, macrophages, eosinophils and neutrophils) and the production of $O_2^{\bullet-}$ is an important factor in the killing of bacteria by phagocytes (Halliwell & Gutteridge, 1985; Packer & Glazer, 1990). In living organisms, $O_2^{\bullet-}$ is removed by the enzymes called superoxide dismutases (SOD).

* Corresponding author. Fax: +1-709-737-4000.
E-mail address: fshahidi@morgan.ucs.mun.ca (F. Shahidi).

0308-8146/00/$ - see front matter
PII: S0308-8146(99)00269-1

The H_2O_2, formed due to four-electron reduction of O_2 into H_2O and dismutation of $O_2^{\bullet-}$, is not a free radical, but an oxidizing agent. In the presence of $O_2^{\bullet-}$ and transition metal ions, H_2O_2 can generate $^{\bullet}OH$ via the superoxide-driven Fenton reaction (Halliwell & Gutteridge, 1985). The $^{\bullet}OH$, formed by this reaction and four electron reduction of O_2, is highly reactive and causes damage to deoxyribonucleic acid (DNA) and initiates lipid oxidation (Packer & Glazer, 1990).

ROS have been implicated in the development and progression of cancer (Slaga, Sirak & Boutwell, 1978) as well as inflammation and aging (Rohrdanz & Kahl, 1998). Frankel and Chrzan (1987) have reported the DNA base modification by H_2O_2 in polymorphonuclear leukocytes. Both DNA-damaging agents (initiating mutagens) and promoters play important roles in carcinogenesis. The toxicity of $O_2^{\bullet-}$ and H_2O_2 in living organisms, however, is due to their conversion into $^{\bullet}OH$ and reactive-radical metal complexes. These processes are often referred to as iron-catalyzed Haber–Weiss reaction. On production in vivo, $^{\bullet}OH$ reacts at its site of formation; thus it has an estimated half-life in cells of only 10^{-9} s (Halliwell & Gutteridge, 1985). In vivo, $^{\bullet}OH$ generation involves radiolysis of water, photolysis of H_2O_2, Fenton reaction and electron transfer mechanisms (Korycka-Dahl & Richardson, 1978).

The occurrence of ROS in foods is inevitable due to the biological nature of foods. Kanner, Harel and Hazan (1986) reported that muscle lipid oxidation is initiated by ROS and haem proteins. ROS also initiate lipid peroxidation in vegetable and animal fats and oils (Bradley & Min, 1992; Rawls & van Santen, 1970). Free radical species of oxygen can directly abstract hydrogen atoms from methylene groups adjacent to olefinic groups of fatty acids resulting in the formation of fatty acid free radicals. In meats, H_2O_2 can generate $^{\bullet}OH$ in the presence of Fe^{2+} via Fenton reaction (Kanner, 1994; Kanner & Doll, 1991; Kanner et al., 1987) causing rapid deterioration of meat lipids.

Many studies have been carried out on biological systems to investigate the preventive action of vitamins E (α-tocopherol), C (L-ascorbic acid) and A on ROS injury. In this respect, vitamin E is most important since it is oxidized by free radicals and regenerated by vitamin C and glutathione (Namiki, 1990). Both vitamins E and C have been reported to deactivate singlet oxygen, $O_2^{\bullet-}$ and $^{\bullet}OH$ in vitro (Bielski, Richeter & Chan, 1976). Studies in animals and in vitro systems have shown that phenolic antioxidants can inhibit free radical-induced damage to macromolecules. Green tea catechins are the most-investigated phenolic antioxidants in this regard (Zhao, He, Cheng & Wenjuan, 1989). The objective of this study was to investigate the ROS-scavenging properties of extracts of borage and evening primrose meals (defatted ground seeds).

2. Materials and methods

Evening primrose and borage seeds were obtained from Scotia Pharmaceuticals Ltd., Kentville, NS, and Bioriginal Food Co. Ltd., Saskatoon, SK, respectively. Three one kilogram packages of each type of seeds were received and stored at −20°C until used. Butylated hydroxyanisole (BHA), mono- and dibasic sodium phosphate, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), nitro blue tetrazolium (NBT), hypoxanthine, xanthine oxidase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), (+)catechin, sinapic acid, Folin-Denis reagent and Sephadex LH-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Hexane, methanol, ethanol, acetone, toluene, *n*-butanol, hydrogen peroxide and ferrous sulphate were obtained from Fisher Scientific (Nepean, ON).

2.1. Preparation of borage and evening primrose crude extracts

Seeds were ground in an electric coffee grinder (Black & Decker Canada Inc., Brockville, ON) for 15 min and then defatted by blending ground seeds with hexane (1:5 w/v, 5 min, ×3) in a Waring Blendor (Model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted seeds were air-dried for 12 h and stored in vacuum-packaged polyethylene pouches at −20°C until used. Extraction of phenolic compounds present in the meals was carried out under reflux conditions in a thermostatted water bath. The antioxidant compounds present in borage meal (6 g) were extracted into 52% (v/v) ethanol at 72°C for 62 min, while those of evening primrose meal (6 g) were extracted into 56% (v/v) acetone at 71°C for 47 min. The optimum extraction conditions for each type of meal were obtained using a response surface methodology (RSM, unpublished data). The resulting slurries were centrifuged for 5 min at 4000×*g* (ICE Centra M5, International Equipment Co., Needham Heights, MA) and the supernatants were collected. The solvent was removed under vacuum at 40°C and the resulting concentrated solutions were lyophilized for 72 h at −49°C and 62×10^{-3} mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO). The content of total phenolics in the extracts as mg catechin (for evening primrose) or sinapic acid (for borage) equivalents/g extract was determined.

2.2. Column chromatographic fractionation of crude extracts

A 1-g portion of crude extracts was dissolved in 10 ml of HPLC grade methanol and applied to a column (1.5 cm diameter and 77 cm height) filled with Sephadex LH-20 (particle size 25–100 mm, Sigma Chemical Co.,

Nepean, ON) and eluted with methanol. Methanolic fractions (8 ml each) were collected in test tubes placed in a LKB Bromma 2112 redirac fraction collector (Pharmacia, Uppsala, Sweden) and their absorbance was measured at 280 nm. Eluates were then pooled into fractions I–VI. Solvent was evaporated under vacuum at 40°C. Dried fractions were stored in tinted glass bottles at −18°C until used. The total phenolics of each fraction were determined, as described in the following section.

2.3. Determination of the content of total phenolics

Extracts were dissolved in methanol to obtain a concentration of 0.5 mg/ml. Folin–Denis reagent (0.5 ml) was added to centrifuge tubes containing 0.5 ml of the extracts. Contents were mixed and 1 ml of a saturated sodium carbonate solution was added into each tube. Volume was then adjusted to 10 ml by the addition of 8 ml of deionized water and the contents were mixed vigorously. Tubes were allowed to stand at ambient temperature for 25 min and then centrifuged for 5 min at $4000\times g$. Absorbance of the supernatants was measured at 725 nm. A blank sample for each extract was used for background subtraction. Content of total phenolics in each extract was determined using a standard curve prepared for (+)catechin or sinapic acid (Swain & Hillis, 1959). Total extracted phenolics were expressed as mg (+)catechin (for evening primrose) or *trans*-sinapic acid (for borage) equivalents/g extract. Evening primrose crude extract produced a pink-coloured complex with an acidic solution of vanillin which indicated the presence of condensed tannins. Therefore, (+)catechin was chosen as a suitable standard for evening primrose. Borage crude extract did not contain condensed tannins as evidenced by a negative vanillin test and thus sinapic acid, a common phenolic acid in oilseeds, was used to quantify the borage phenolics.

2.4. Determination of the content of hydrophilic and hydrophobic phenolics

The crude extract was fractionated into its hydrophilic and hydrophobic components by mixing 5 g of it with 100 ml of deionized water and 100 ml of *n*-butanol in a 250 ml separatory funnel. The mixture was allowed to stand at 4°C for 12 h; separated layers were removed and desolventized using a Rotavapor (Buchi, Flawil, Switzerland) set at 40°C. The resulting concentrated solution was lyophilized for 72 h at −49°C and 62×10^{-3} mbar. Weight of each fraction was recorded and the content of phenolics determined.

2.5. Hydrogen peroxide-scavenging assay

Borage and evening primrose crude extracts were dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 μl of a 43 mM solution of hydrogen peroxide (prepared in the same buffer). Authentic sinapic acid and (+)catechin were used as the reference antioxidants. Final concentration of extracts and standards was 100 or 200 ppm. The absorbance values (at 230 nm) of the reaction mixtures were recorded at 0 min and then at every 10 min up to 40 min. For each concentration, a separate blank sample (devoid of hydrogen peroxide) was used for background subtraction (Ruch, Cheng & Klauring, 1989). The concentration (mM) of hydrogen peroxide in the assay medium was determined using a standard curve and hydrogen peroxide-scavenging capacities of additives were calculated using the following equation:

$$\text{Hydrogen peroxide-scavenging capacity, \%} = 100 - (\text{hydrogen peroxide concentration of medium containing the additive of concern/hydrogen peroxide concentration of the control medium}) \times 100$$

2.6. Hydroxyl radical-scavenging assay

The hydroxyl radicals were generated via iron-catalyzed Haber–Weiss reaction (Fenton driven Haber–Weiss reaction) and spin-trapped with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The resultant DMPO-OH adduct was detected using an electron paramagnetic resonance (EPR) spectrometer (Bruker ESP 300, Bruker Instruments, Inc., Billeria, MA). Borage and evening primrose crude extracts as well as authentic sinapic acid and catechin were dissolved in 0.1 M phosphate buffer (pH 7.4) so that a 200 μl aliquot will result in 200 ppm of phenolics in the final assay medium (final volume was 800 μl). For 100 ppm concentration, 100 μl of the same extract stock solution was used, but the volume was adjusted to 200 μl by adding 100 μl of the buffer. Extracts (200 μl) were mixed with 200 μl of 100 μM DMPO, 200 μl of 10 μM ferrous sulphate and 200 μl of 10 μM hydrogen peroxide. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After 3 min, 10 μl of the mixture were drawn into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 2×10^5 receiver gain, 1.0 G modulation amplitude, 200 s scan time, 3460 G centre field, 100 G sweep width and 0.5 s time constant (Shi, Dalal & Jain, 1991). Hydroxyl radical-scavenging capacities of the additives were calculated using the following equation:

$$\text{Hydroxyl radical-scavenging capacity, \%} = 100 - (\text{EPR signal intensity for medium containing the additive of concern/EPR signal intensity for the control medium}) \times 100$$

2.7. Superoxide radical-scavenging assay

A modified version of the method explained by Nishikimi, Rao and Yagi (1972) was employed. Superoxide radicals were generated with an enzymatic reaction. The reaction mixture contained 1 ml of 3 mM hypoxanthine, 1 ml of xanthine oxidase (100 mIU), 1 ml of 12 mM diethylenetriaminepentaacetic acid, 1 ml of 178 mM nitro blue tetrazolium and 1 ml of the extracts (final concentration of the phenolics in the reaction mixture was 200 ppm). For 100 ppm concentration, 0.5 ml of the stock extract solution was diluted with 0.5 ml of the buffer. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). Authentic sinapic acid and catechin (100 and 200 ppm) were used as the reference antioxidants. The absorbance values (at 560 nm) of systems were recorded at 0 min and then after every 10 min up to 60 min. For each system, the absorbance values were corrected by subtracting the 0 min readings from subsequent readings. Superoxide radical-scavenging capacities (after 10 min of assay) of the additives were calculated using the following equation:

$$\text{Superoxide radical-scavenging capacity, } \% = 100 - \left(\text{absorbance of medium containing the additive of concern/absorbance of the control medium}\right) \times 100$$

2.8. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay

One hundred microlitres of a 30 μM solution of DPPH in toluene were added to 100 μl of a solution containing borage or evening primrose crude extracts in toluene so that the concentration of phenolics in the final assay media was either 100 or 200 ppm. Contents were mixed and transferred into an EPR cell. After 60 s, 10 μl of the mixture were drawn into a syringe and transferred into a quartz capillary tube. The spectrum was recorded in the EPR spectrometer set at 2×10^5 receiver gain, 1.0 G modulation amplitude, 200 s scan time, 3460 G centre field, 100 G sweep width and 0.5 s time constant (Santiago, Miramatsu & Mori, 1992). Authentic sinapic acid and catechin (100 and 200 ppm) were used as reference antioxidants. DPPH radical-scavenging capacities of the additives were calculated using the following equation:

$$\text{DPPH radical-scavenging capacity, } \% = 100 - (\text{EPR signal intensity for medium containing the additive of concern/EPR signal intensity for the control medium}) \times 100$$

2.9. Statistical analysis

All experiments, except EPR spectroscopic analyses, used completely randomized block designs (CRD) and analyses were carried out in triplicate. The significance of differences among mean values was determined at $P \leqslant 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple range test (Snedecor & Cochran, 1980).

3. Results and discussion

3.1. Sephadex LH-20 column chromatography of crude extracts of borage and evening primrose meals

Sephadex LH-20 column chromatography has been used by many researchers to fractionate various plant extracts (Amarowicz, Koslowska & Shimoyamada, 1992; Amarowicz & Shahidi, 1996; Amarowicz, Wanasundara, Wanasundara & Shahidi, 1993). Sephadex LH-20 is probably one of the best stationary phases available for separation of phenolics because of the faster, yet satisfactory separation of phenolics on the column (Wanasundara, Amarowicz & Shahidi, 1994). Fig. 1A shows the fraction profile for borage. Even though the profile was continuous, six major fractions were clearly identifiable. These fractions were labelled I–VI. Yield of individual fractions as a relative fraction of the crude extract is given in Table 1. As depicted in Fig. 1B, evening primrose crude extract was also separated into six major fractions (I–VI) with varying relative yields as given in Table 1.

3.2. Content of total, hydrophilic and hydrophobic phenolics in borage and evening primrose crude extracts and their fractions

Table 2 shows the total, hydrophilic and hydrophobic phenolics contents of borage crude extract and its fractions. The total phenolics content of borage crude extract was 413 mg as sinapic acid equivalents/g and it consisted of 89% (w/w) and 11% (w/w) of hydrophilic and hydrophobic phenolics (ratio of 8:1, w/w), respectively. The hydrophilic phenolics contents of borage fractions I–VI were 2.5–6 times higher than their hydrophobic counterparts [approximate ratios of hydrophilic to hydrophobic phenolics for borage fractions I–VI were 2.5:1, 6:1, 3:1, 4.5:1, 3.6:1 and 5:1 (w/w), respectively]. Evening primrose crude extract contained 304 mg of phenolics as catechin equivalents/g and its hydrophilic and hydrophobic phenolics were present at a 3:2 (w/w) ratio (Table 3). Almost equal amounts of hydrophilic and hydrophobic phenolics were present in evening primrose fractions I and V. Fraction II had a somewhat higher content of hydrophobic phenolics as

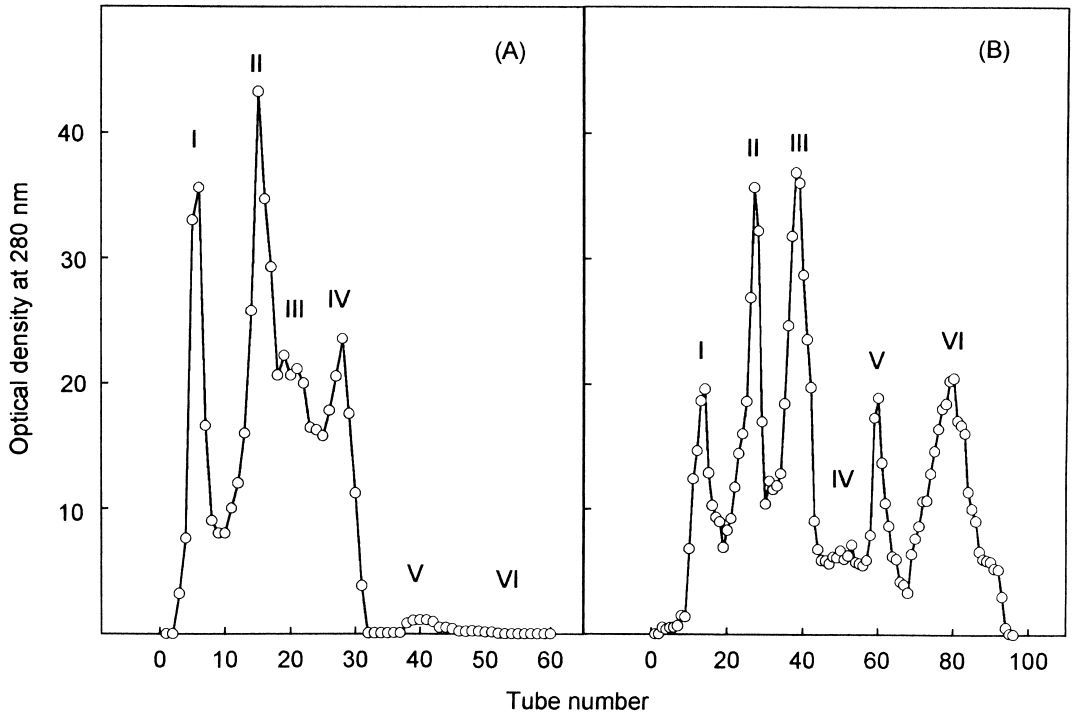

Fig. 1. Column chromatographic fraction profiles for borage (A) and evening primrose (B).

compared to its hydrophilic counterpart. Fraction III had two times more hydrophobic phenolics than hydrophilic, and the reverse was evident for fraction VI. The hydrophobic phenolics of fraction IV were 35 times higher than hydrophilic phenolics [Approximate ratios of hydrophilic to hydrophobic phenolics for evening primrose fractions I–VI were 1:1, 1:1.4, 1:2, 1:35, 1:1, and 2:1 (w/w), respectively]. These results show the presence of varying amounts of both hydrophilic and hydrophobic phenolics in borage and evening primrose crude extracts and their fractions.

Table 1
Column chromatoraphic data for borage and evening primrose[a]

Fraction	Borage[b]		Evening primrose[c]	
	Weight (mg)	Relative fraction (%, w/w)	Weight (mg)	Relative fraction (%, w/w)
I	168	19.6	162	17.3
II	309	36.2	218	23.2
III	244	28.4	287	30.6
IV	65.0	7.57	70.2	7.46
V	26.4	3.07	65.3	6.93
VI	46.0	5.35	136	14.5

[a] Results are averages of two runs (within 7%).
[b] Weight of crude extract used = 1 g; recovery = 86%.
[c] Weight of crude extract used = 1 g; recovery = 94%.

3.3. Hydrogen peroxide (H_2O_2)-scavenging capacity of borage and evening primrose crude extracts and their fractions

Fig. 2 depicts the H_2O_2-scavenging capacity of 100 and 200 ppm of borage additives; values were lower for 100 ppm as compared to those for 200 ppm level of addition. At 100 ppm, none of the additives could completely remove H_2O_2 from the assay medium (about 10–90% scavenging) but, at 200 ppm, borage fractions III, IV and V scavenged H_2O_2 completely. Meanwhile, 200 ppm sinapic acid used as the reference antioxidant, scavenged H_2O_2 completely. For both concentrations, borage fraction VI was the least effective among borage additives while other borage additives, at 200 ppm, scavenged 60–75% of H_2O_2.

Hydrogen peroxide-scavenging capacity of evening primrose additives, at 100 and 200 ppm, are depicted in Fig. 2. Evening primrose crude extract and its fraction I, at 100 ppm, showed weak H_2O_2-scavenging capacities. At 200 ppm, the efficacy of crude extract was enhanced, but fraction I remained less efficient. Hydrogen peroxide-scavenging capacities of 200 ppm of evening primrose crude extract and fractions II–V were similar. Catechin, at both concentrations, was highly efficient in its H_2O_2-scavenging effect.

Scavenging of H_2O_2 by borage and evening primrose additives may be attributed to their phenolics which could donate electrons to H_2O_2, thus neutralizing it to water, as shown in the following equation (Halliwell & Gutteridge, 1985).

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$$

Table 2
Contents of total, hydrophilic and hydrophobic phenolics of borage crude extract and its fractions[a]

Sample	Phenolics as mg sinapic acid equivalents/g sample		
	Total	Hydrophilic[b]	Hydrophobic[b]
Crude extract	413 ± 18	367 ± 16 (89%)	46 ± 2 (11%)
Fraction I	283 ± 16	200 ± 11 (70%)	83 ± 4 (30%)
Fraction II	129 ± 5	111 ± 5 (86%)	18 ± 2 (14%)
Fraction III	140 ± 8	106 ± 7 (76%)	34 ± 2 (24%)
Fraction IV	366 ± 14	300 ± 12 (82%)	66 ± 3 (18%)
Fraction V	280 ± 10	220 ± 10 (78%)	60 ± 3 (22%)
Fraction VI	347 ± 15	290 ± 15 (83%)	57 ± 3 (17%)

[a] Results are mean values of three determinations ± standard deviation.
[b] Values in parentheses show the % (w/w) contents of hydrophilic and hydrophobic phenolics in the total phenolics.

Table 3
Contents of total, hydrophilic and hydrophobic phenolic of evening primrose crude extract and its fractions[a]

Sample	Phenolics as mg catechin equivalents/g sample		
	Total	Hydrophilic[b]	Hydrophobic[b]
Crude extract	304 ± 14	185 ± 7 (61%)[b]	121 ± 6 (39%)
Fraction I	158 ± 10	78 ± 5 (49%)	80 ± 6 (51%)
Fraction II	313 ± 18	128 ± 10 (41%)	185 ± 10 (59%)
Fraction III	369 ± 20	123 ± 9 (33%)	246 ± 12 (67%)
Fraction IV	402 ± 26	11 ± 1 (3%)	391 ± 23 (97%)
Fraction V	279 ± 10	136 ± 8 (49%)	143 ± 4 (51%)
Fraction VI	445 ± 25	300 ± 15 (67%)	145 ± 8 (33%)

[a] Results are mean values of three determinations ± standard deviation.
[b] Values in parentheses show the % (w/w) contents of hydrophilic and hydrophobic phenolics in the total phenolics.

The differences in H_2O_2-scavenging capacities of borage and evening primrose additives may be attributed to the structural features of their active components which determine their electron donating abilities.

3.4. Free radical-scavenging capacity of borage and evening primrose crude extracts and their fractions

Superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), two oxygen-derived free radical species, and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), an organic free radical, were used to study the free radical-scavenging capacity of borage and evening primrose crude extracts and their fractions. The first species investigated was $O_2^{\bullet-}$, generated in a hypoxantine/xanthine oxidase system. The $O_2^{\bullet-}$ was readily scavenged by borage and evening primrose additives (Fig. 3). A complete scavenging of $O_2^{\bullet-}$ was evident for assay media containing 200 ppm borage and evening primrose additives. The only exceptions were borage fraction V and evening primrose

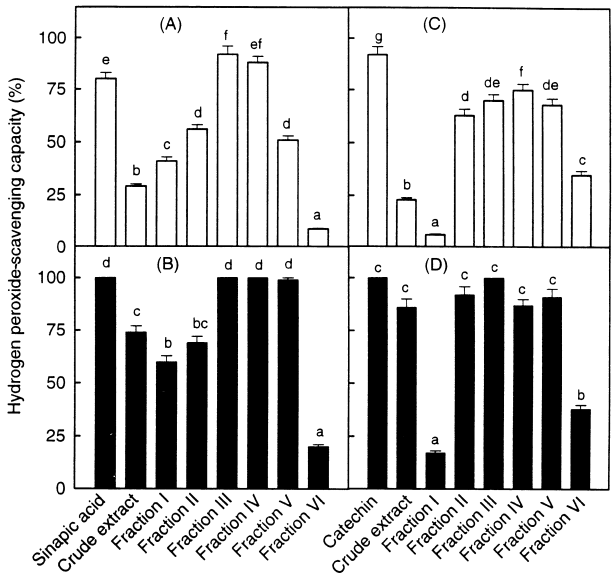

Fig. 2. Hydrogen peroxide-scavenging capacities of borage and evening primrose crude extracts and their fractions. (A) and (B) depict the results for 100 and 200 ppm borage crude extract/fractions, respectively. (C) and (D) depict the results for 100 and 200 ppm evening primrose crude extract/fractions, respectively. Each bar represents the mean value of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly different ($P > 0.05$) from one another.

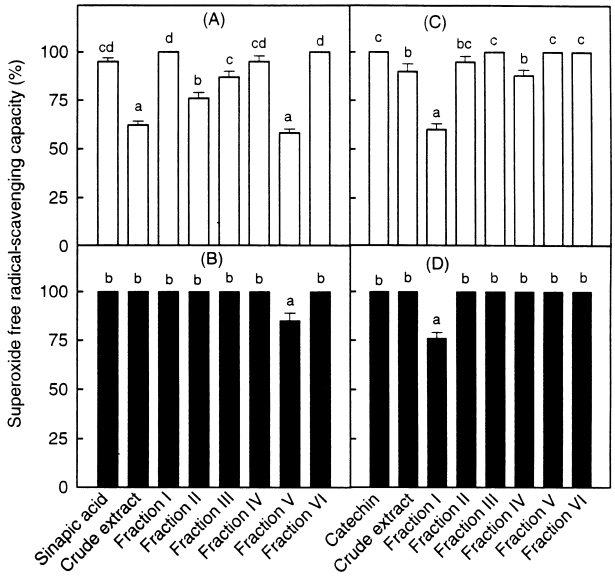

Fig. 3. Superoxide radical-scavenging capacities of borage and evening primrose crude extracts and their fractions. (A) and (B) depict the results for 100 and 200 ppm borage crude extract/fractions, respectively. (C) and (D) depict the results for 100 and 200 ppm evening primrose crude extract/fractions, respectively. Each bar represents the mean value of three determinations ± standard deviation. Bars sharing the same letter in a group of bars are not significantly different ($P > 0.05$) from one another.

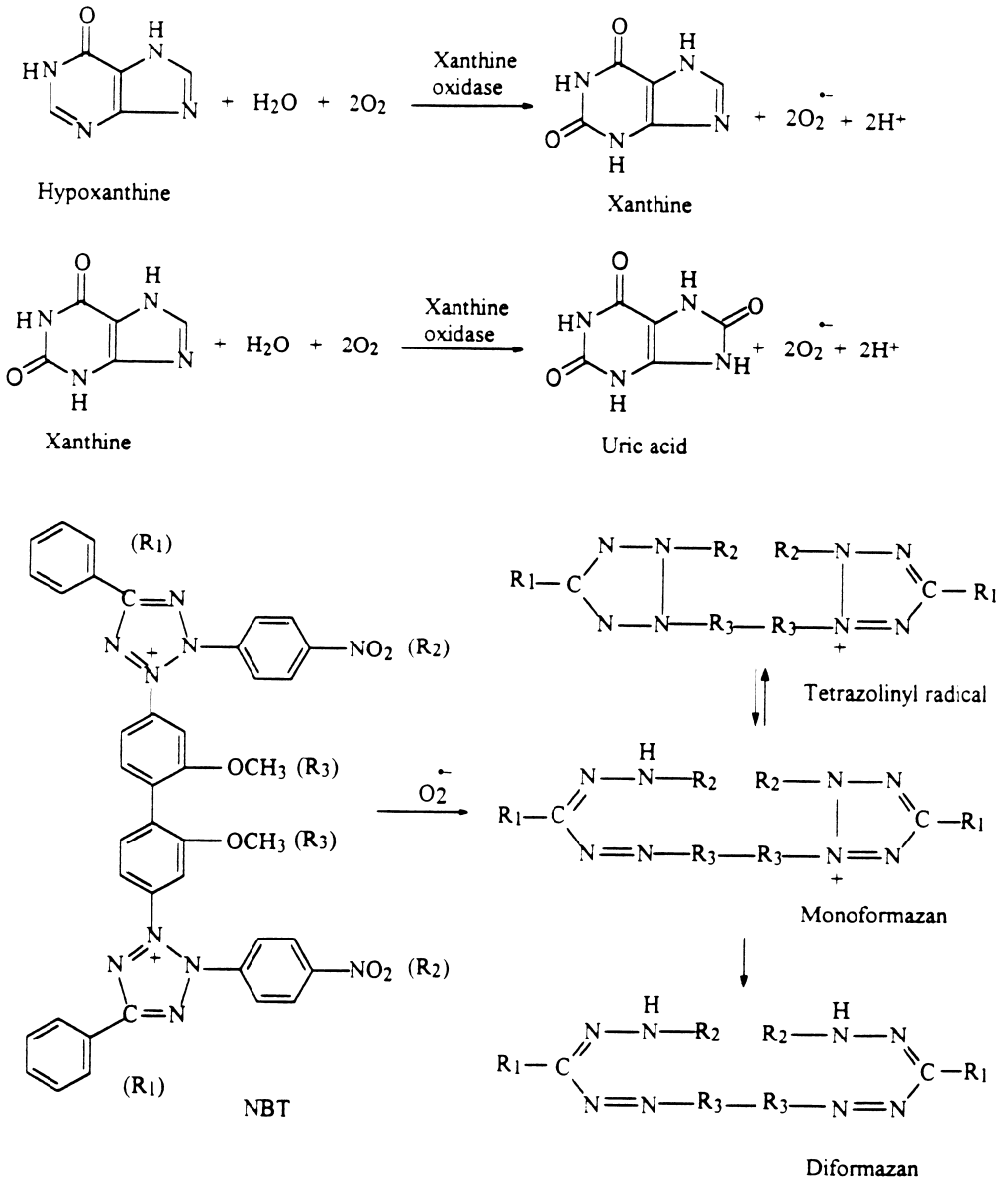

Fig. 4. Mechanism of ink-blue colour generation in the superoxide-scavenging assay.

fraction I which exhibited approximately 75% scavenging of $O_2^{\bullet-}$. For the control and 100 and 200 ppm of some additives, the generation of $O_2^{\bullet-}$ was indicated by the development of an ink-blue colour in the assay media; the mechanism of the colour generation is depicted in Fig. 4. For the control, intensity of this colour increased with time, but then leveled off. Therefore, the absorbance values (at 560 nm) after 60 min were used to determine the $O_2^{\bullet-}$-scavenging capacity of the additives. The scavenging of $O_2^{\bullet-}$ by additives may be attributed to their various phenolic constituents. Cotelle, Bernier, Catteau, Pommery, Wallet and Gaydou (1996) reported that flavones, a class of flavonoids, bearing hydroxyl groups at positions 3′, 4′ or 3′, 4′, 5′ scavenged $O_2^{\bullet-}$. These authors also reported that flavonoids containing one hydroxyl group in the C-7 position of ring A could inhibit generation of $O_2^{\bullet-}$ in the xanthine/xanthine oxidase system by inhibiting the enzyme. Yuting, Rongliang, Zhonjiang and Yong (1990) reported that different types of flavonoids, namely rutin, naringin, quercetin and hispidulin, scavenged $O_2^{\bullet-}$. Several researchers have suggested that a 3′,4′-diphenolic group on ring B is required for flavonoids to be effective free radical scavengers (Latan, 1966; Younes & Siegers, 1981). Phenolic acids and their derivatives are also reported to be excellent free radical-scavengers. Rice-Evans, Miller and Paganga (1996) reported that dihydroxybenzoic acids, such as protocatechuic and resorcylic acids, as well as hydroxycinnamic acids, such as caffeic and ferulic acids, were able to quench free radicals, including $O_2^{\bullet-}$, by hydrogen donation. The radical-scavenging ability of phenolic acids is mediated by the

number of hydroxyl groups in the molecule (Dziedzic & Hudson, 1983). The electron-withdrawing property of the carboxylic acid group in benzoic acid has a negative influence on hydrogen-donating ability of hydroxybenzoates. Hydroxylated cinnamates are more effective than their benzoate counterparts (Rice-Evans et al.). Therefore, $O_2^{\bullet-}$-scavenging properties of borage and evening primrose additives may also be attributed to both neutralization of superoxide radicals via hydrogen donation and inhibition of xanthine oxidase by various phenolic components present in the additives.

Hydroxyl radicals were generated through an iron-catalyzed Haber–Weiss reaction as shown below.

$$Fe^{2+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \text{ (metal reduction)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \text{ (Fenton reaction)}$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^- \text{ (Haber–Weiss reaction)}$$

Hydroxyl radicals, generated via these reactions, were spin-trapped with DMPO (5,5-dimethyl-1-pyrrole-*N*-oxide) which formed a DMPO-OH adduct, a relatively stable free radical (Halliwell & Gutteridge, 1985; Ruch et al., 1989). Spin-trapping was done because the detection of hydroxyl radicals, as such, is extremely difficult due to their very short life time (Ruch et al.). As shown in Fig. 5, the DMPO-OH adduct generated a 1:2:2:1 quartet with hyperfine coupling constant of 14.9 G (Yen & Chen, 1995).

The characteristic quartet signal for DMPO-OH adduct was not detected in the assay media containing 100 and 200 ppm sinapic acid. One possibility was the quenching of $^{\bullet}OH$ by sinapic acid due to hydrogen donation. The other possibility was inhibition of generation of $^{\bullet}OH$ due to chelation of iron (II) by sinapic acid. For 100 ppm borage fractions IV and VI, the characteristic quartet signal for DMPO-OH adduct was not detected. At 200 ppm, the signal was detected only in assay medium containing borage fraction III while the signal was not detected in assay media containing other additives. Reduction in signal intensity (scavenging capacity) due to the presence of 100 and 200 ppm borage additives in the assay media are depicted in Fig. 6A and 6B, respectively. Signal intensity for the control was 100%; thus its scavenging capacity was zero (not shown in Fig. 6).

The EPR signal of DMPO-OH adduct was not detected in assay media containing 100 ppm of catechin and evening primrose (concentrations based upon phenolics as catechin equivalents) fractions II, III and VI. No signal was detected when catechin and evening primrose additives were present at 200 ppm concentration. Reductions in signal intensity (scavenging capacity) due to the presence of 100 and 200 ppm evening primrose additives in the assay media are depicted in Fig. 6C and 6D, respectively. The disappearance of the EPR signal in assay media containing evening primrose additives may also be attributed to the same reasons as explained for borage additives.

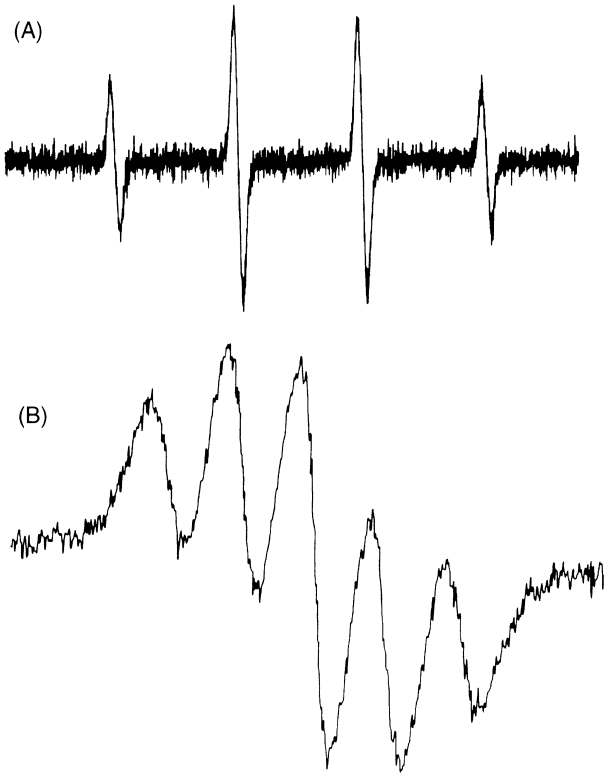

Fig. 5. Electron paramagnetic resonance spectrum of DMPO-OH adduct (A) and DPPH free radical (B) detected in the control assay medium.

DPPH$^{\bullet}$, a stable free radical, has been used to evaluate free radical-scavenging capacities of natural antioxidants (Blois, 1958). Unlike the laboratory generated free radicals, such as $O_2^{\bullet-}$ and $^{\bullet}OH$, use of a stable free radical has the advantage of being unaffected by side reactions, such as metal chelation and enzyme inhibition, brought about by the additives. The reduction of DPPH$^{\bullet}$ in the presence of an additive was monitored by measuring the intensity of EPR signals. Generalized reduction reactions between DPPH$^{\bullet}$ and additives may be written as:

$$DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}$$

$$DPPH^{\cdot} + R^{\cdot} \rightarrow DPPH - R$$

where AH and R$^{\bullet}$ denote antioxidant components of the additives and radical species, respectively. Fig. 5 shows the EPR spectrum for DPPH$^{\bullet}$. Fig. 7A–D depicts the reduction in signal intensity (scavenging capacity) in the presence of borage and evening primrose additives.

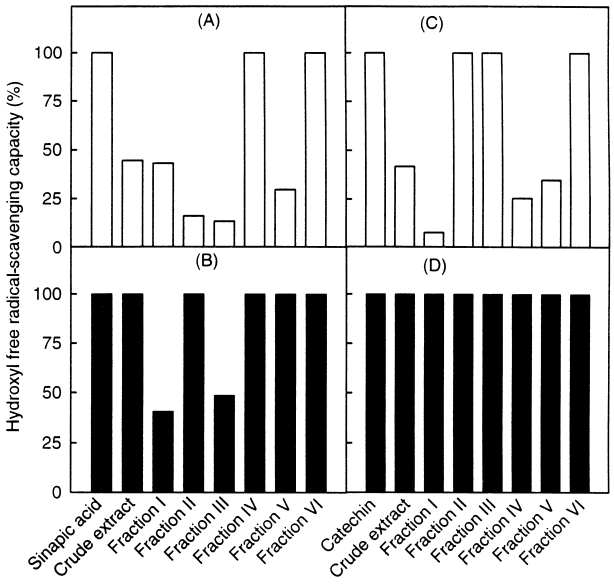

Fig. 6. Hydroxyl radical-scavenging capacity of borage and evening primrose crude extracts and their fractions. (A) and (B) depict the results for 100 and 200 ppm borage crude extract/fractions, respectively. (C) and (D) depict the results for 100 and 200 ppm evening primrose crude extract/fractions, respectively.

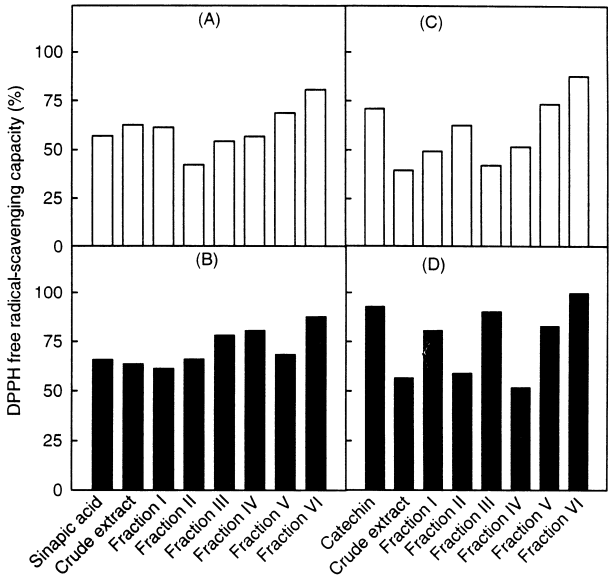

Fig. 7. DPPH radical-scavenging capacity of borage and evening primrose crude extracts and their fractions. (A) and (B) depict the results for 100 and 200 ppm borage crude extract/fractions, respectively. (C) and (D) depict the results for 100 and 200 ppm evening primrose crude extract/fractions, respectively.

The control exhibited 100% signal intensity; thus its scavenging capacity was zero (not shown in Fig. 7). Borage additives, at 100 ppm, scavenged DPPH$^{\bullet}$ by about 42–88%. Scavenging capacity for evening primrose additives ranged from 40 to 100%. These results suggest that both borage and evening primrose additives possess the ability to quench free radicals and the effects may be attributed to the hydrogen and electron donating abilities of their phenolics.

4. Conclusions

Borage and evening primrose crude extracts and their fractions, separated via a column chromatographic procedure, possess strong ROS- and DPPH$^{\bullet}$-scavenging properties which are either comparable or superior to those of authentic catechin and sinapic acid at the same concentration. These effects are attributable to varying hydrogen-donating and metal-chelating capacities of the phenolic compounds present in the crude extracts/fractions. Borage and evening primrose extracts and their fractions may be incorporated into seed oils and other types of oils and lipid-containing foods as chain-breaking antioxidants to minimise free radical-mediated lipid peroxidation. They may also be used as alternative drugs to treat human diseases associated with free radical-mediated tissue damage. Such uses, however, have to be adequately justified using animal and clinical studies; hence further research is needed in this regard.

Acknowledgements

We are thankful to the Natural Sciences and Engineering Research Council of Canada for financial support.

References

Amarowicz, R., Koslowska, H., & Shimoyamada, M. (1992). Chromatographic analysis of rapseed glucosides fractions. *Polish Journal of Food and Nutrition, 1*, 89–93.

Amarowicz, R., Wanasundara, U., Wanasundara, J., & Shahidi, F. (1993). Antioxidant activity of ethanolic extracts of flaxseed in a β-carotene-linoleate model system. *Journal of Food Lipids, 1*, 111–117.

Amarowicz, R., & Shahidi, F. (1996). A rapid chromatographic method for separation of individual catechins from green tea. *Food Research International, 29*, 71–76.

Bielski, B. H. J., Richeter, H. W., & Chan, P. C. (1976). Some properties of the ascorbate free radical. *Annals of New York Academy of Science, 258*, 231–333.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Science, 181*, 1199–1200.

Borg, D. C. (1993). Oxygen free radicals and tissue injury. In M Tarr, & F. Samson, *Oxygen free radicals in tissue damage* (pp. 12–55). Boston, MA: Birkhauser.

Bradley, D. G., & Min, D. B. (1992). Singlet oxygen oxidation of foods. *Critical Reviews in Food Science and Nutrition, 31*, 211–236.

Cotelle, N., Bernier, J.-L., Catteau, J.-P., Pommery, J., Wallet, J.-C., & Gaydou, E. M. (1996). Antioxidant properties of hydroxyflavones. *Free Radical Biology and Medicine, 20*, 35–43.

Dziedzic, S. Z., & Hudson, B. J. F. (1983). Polyhydroxychalcones and flavanones as antioxidants for edible foods. *Food Chemistry, 12*, 205–212.

Frankel, K., & Chrzan, K. (1987). Hydrogen peroxide formation and DNA base modification by tumor promotor-activated polymorphonuclear leukocytes. *Carcinogenesis, 8*, 455–460.

Halliwell, B., & Gutteridge, J. M. C. (1985). *Free radicals in biology and medicine*. Oxford, UK: Oxford University Press.

Kanner, J. (1994). Oxidative processes in meat and meat products: quality implications. *Meat Science, 36*, 169–189.

Kanner, J., & Doll, L. (1991). Ferritin in turkey muscle tissue: a source of catalytic iron ions for lipid peroxidation. *Journal of Agricultural and Food Chemistry, 39*, 247–249.

Kanner, J., German, J. B., & Kinsella, J. E. (1987). Initiation of lipid peroxidation in biological systems. *Critical Reviews in Food Science and Nutrition, 25*, 317–364.

Kanner, J., Harel, S., & Hazan, B. (1986). Muscle membranal lipid peroxidation by an ''iron redox cycle'' system. Initiation by oxy-radicals and site specific mechanisms. *Journal of Agricultural and Food Chemistry, 34*, 506–510.

Korycka-Dahl, M. B., & Richardson, T. (1978). Activated oxygen species and oxidation of food constituents. *Critical Reviews in Food Science and Nutrition, 10*, 209–242.

Latan, A. (1966). The relation of structure to antioxidant activity of quercetin and some of its derivatives: 1. Primary activity. *Journal of Food Science, 31*, 395–399.

Namiki, M. (1990). Antioxidants/antimutagens in food. *Critical Reviews in Food Science and Nutrition, 29*, 273–300.

Nishikimi, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochemical Biophysical Research Communications, 46*, 849–854.

Packer, L. (1994). *Methods in enzymology: oxygen radicals in biological systems* (Vol. 233, Part C). San Diego, CA: Academic Press.

Packer, L., & Glazer, A. N. (1990). *Methods in enzymology: oxygen radicals and antioxidants* (Vol. 186, Part B). San Diego, CA: Academic Press.

Rawls, H. R., & van Santen, J. P. (1970). A possible role for singlet oxidation in the initiation of fatty acid autoxidation. *Journal of the American Oil Chemists' Society, 47*, 121–124.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Rohrdanz, E., & Kahl, R. (1998). Alterations of antioxidant enzyme expression in response to hydrogen peroxide. *Free Radical Biology and Medicine, 24*, 27–38.

Ruch, R. J., Cheng, S.-T., & Klauring, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis, 10*, 1003–1008.

Santiago, L. A., Hiramatsu, M., & Mori, A. (1992). Japanese soybean paste miso scavenges free radicals and inhibits lipid peroxidation. *Journal of Nutritional Science and Vitaminology, 38*, 297–304.

Shi, X., Dalal, N. S., & Jain, A. C. (1991). Antioxidant behaviour of caffeine: efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology, 29*, 1–6.

Slaga, J., Sirak, A., & Boutwell, R. K. (1978). *Carcinogenesis. Mechanism of tumor promotion and carcinogenesis* (Vol. 2). New York: Raven Press.

Snedecor, G. W., & Cochran, W. G. (1980). *Statistical Methods* (7th ed.). Ames, IA: Iowa State University Press.

Swain, T., & Hillis, W. E. (1959). The phenolic constituents of Prunes domesticus (L.). The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture, 10*, 63–68.

Wanasundara, U., Amarowicz, R., & Shahidi, F. (1994). Isolation and identification of an antioxidative component in canola meal. *Journal of Agricultural and Food Chemistry, 42*, 1285–1290.

Yen, G.-C., & Chen, H.-Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenecity. *Journal of Agricultural and Food Chemistry, 43*, 27–32.

Younes, M., & Siegers, C. P. (1981). Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion. *Planta Medicine, 43*, 240–243.

Yuting, C., Rongliang, Z., Zhonjiang, J., & Yong, J. (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biology and Medicine, 9*, 19–21.

Zhao, B., Li, X., He, R.-G., Cheng, S.-J., & Wenjuan, X. (1989). Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. *Cell Biophysiology, 14*, 175–179.