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Chemical Composition and in Vitro Antioxidant Evaluation of Commercial Water-Soluble Willow Herb (*Epilobium angustifolium* L.) Extracts

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Five commercially available water-soluble extracts prepared from the aerial parts of *Epilobium angustifolium* L. (Onagraceae) were screened for antioxidant-related properties in a battery of six in vitro assays. Total phenol content and qualitative−quantitative analyses were also carried out. The extracts demonstrated varying degrees of efficacy in each screen. Two extracts, denoted as nonfermented and Tver, were the most effective toward reducing iron(III), scavenging 1,1-diphenyl-2-picrylhydrazyl free radicals, inhibiting hydroxyl radical-catalyzed bovine brain-derived phospholipid degradation, and non-site- and site-specific hydroxyl radical-catalyzed 2-deoxy-D-ribose degradation. The activity profile of the extracts changed, however, when their iron(II) chelating ability was assessed. The nonfermented and Tver extracts were not as effective iron(II) chelators as the extract denoted as Lotos. All the extracts contained Folin−Ciocalteu-reactive substances, which was confirmed by the presence of predominantly polar phenolic analytes (i.e. hydroxylated benzoic acid derivatives and flavonoids).

KEYWORDS: Antioxidants; *Epilobium angustifolium* L.; willow herb; high performance liquid chromatography; polyphenols; 1,1-diphenyl-2-picrylhydrazyl (DPPH•); free radicals; chelation; Onagraceae; water-soluble extracts.

INTRODUCTION

Interest in plant-derived antioxidants has grown exponentially in the last couple of decades. The principal reasons for this appears to be (i) the recognition that oxidative stress plays a cardinal role in the etiology of chronic diseases such as atherosclerosis, cancer, diabetes, and age-related neurological degenerative diseases among others (*1*−*3*); (ii) the clarification of the beneficial role antioxidant-rich diets have upon the expression of such disease states (*4*); and (iii) the confusion regarding the safety of the chronic consumption of synthetic preservatives traditionally used in foods and beverages (*5*, *6*). Compounds thought to be primarily responsible for the health-promoting properties of diets heavily based on fruit, berry, cereal, vegetable, and certain beverage consumption are hydroxycinnamates (*7*, *8*) and flavonoids (*9*, *10*).

The genus *Epilobium* (Onagraceae) consists of ca. 200 species and is distributed throughout the world. In Russia, approximately sixty *Epilobium* species have been described, the most common being willow herb (*E. angustifolium* L., syn. *Chamaenerion angustifolium* L.). Traditionally, willow herb has been used in folk medicine to treat a variety of ailments such as benign prostate hyperplasia and the associated problems of micturition (*11*, *12*). American Indians are documented as using it for rectal bleeding, while the Chinese have used it for the relief of menstrual disorders. In Russia, it is usually consumed as either fermented or nonfermented infusions (commonly referred to as Ivan tea) for the treatment of stomach ulceration, gastritis, and sleeping disorders. The consumption of these teas is very common due to the high prevalence of these diseases in the population (*13*, *14*). In vivo, aqueous extracts from *Epilobium* taxa have been ascribed a variety of beneficial properties, including analgesic, antiinflammatory, antitumor, and antiandrogenic properties (*15*−*17*). Ethanolic extracts from *E. angustifolium* have demonstrated bactericidal activity against both Gram-types of bacteria and cytocidal activity against fungi (*18*). Information regarding the chemical composition of *Epilobium* species in the scientific literature is limited (*12*, *19*), as is the characterization of their antioxidant properties. Thus, as part of our ongoing phytochemical and in vitro antioxidant characterization of herbs, spices, and medicinal plant-based preparations, we have investigated the qualitative−quantitative chemical

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composition and antioxidant properties of commercially available water-soluble extracts isolated from the aerial parts of *E. angustifolium* collected from five different geographical locations. The data presented should make a further contribution to the current knowledge regarding the chemistry and functional properties of one member of this important, yet underinvestigated, genus.

MATERIALS AND METHODS

Materials. Water-soluble *E. angustifolium* extracts (Diod, Express-service, Lotos, nonfermented, and Tver) were obtained from Diod Ltd. (Moscow, Russia). Chromatography standards were purchased from either Extrasynthase (Genay, France) or Sigma Chemical Co. (St. Louis, MO). Ultrapure water (18.2 MΩ cm) was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., Bedford, MA). All reagents and solvents were of either analytical or HPLC grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Dry Extracts. The botanical material was dried in a dehydration tunnel at 40 °C for 5−6 h. After comminution, the tea was placed in heaps on outside platforms, wetted and bruised to initiate "fermentation", and fermented for 6 h at 40−41 °C (except the unfermented sample). Fermented teas were sampled from the heaps and dried in a dehydration tunnel at 40 °C for 4−6 h. The material obtained was suspended in water and extracted by a vacuum-pulsation procedure for 2 h (*20*). The resulting aqueous extracts were then filtered, reduced in volume in vacuo (45 °C), dried, and stored at 4 °C.

Total Phenols. The total phenol content of the extracts was estimated as gallic acid equivalents, expressed as mg of gallic acid/g (dry wt) of extract, according to the Folin−Ciocalteu reagent method (*21*). Briefly, 0.25 mL of sample was transferred to a 25.0 mL volumetric flask containing 6 mL of H_2O, to which was added 1.25 mL of undiluted Folin−Ciocalteu reagent. After 1 min, 3.75 mL of 20% (w/v) aqueous Na_2CO_3 was added, and the volume was made up to 25.0 mL with H_2O. The controls contained all the reaction reagents except the extract. After a 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. Data are calculated as mean values ± standard deviation ($n = 3$).

Chromatographic Fingerprint Analyses. The liquid chromatographic apparatus (Waters 600) consisted of an in-line degasser, pump, and controller coupled to a 2996 photodiode array detector equipped with a Rheodyne injector (20 μL sample loop) interfaced to a PC running Millenium^{32} chromatography manager software (Waters Corp., Milford, MA). Separations were performed on a reverse-phase Hypersil BDS-C18 analytical column (250 × 4.6 mm i.d., particle size 5 μm) (Agilent Technologies, Milford, MA) operating at room temperature with a flow rate of 0.7 mL/min. Detection was carried out with a sensitivity of 0.1 aufs. between the wavelengths of 200 and 550 nm. Elution was effected using a ternary nonlinear gradient of the solvent mixture MeOH/H_2O/CH_3COOH (10:88:2, v/v/v) (solvent A), MeOH/H_2O/CH_3COOH (90:8:2, v/v/v) (solvent B), and MeOH (solvent C). The composition of the mobile phase was changed from 99:1:0 (A/B/C) to 98:2:0 (A/B/C) in 15 min, to 60:40:0 (A/B/C) in 3 min, to 50:50:0 (A/B/C) in 12 min, to 5:95:0 (A/B/C) in 5 min, to 0:85:15 (A/B/C) in a further 2 min, to 0:70:30 (A/B/C) in 11 min, and then returned to initial conditions in 7 min. A 10 min equilibrium time was allowed between injections.

The components were identified by comparison of their retention times to those of authentic standards under identical analysis conditions and the UV spectra with our in-house PDA library.

Chromatographic Standards and Sample Preparation. Stock solutions of the extracts and standards were prepared in water and 70% (v/v) aqueous methanol to final concentrations of 1 and 10 mg/mL, respectively. The concentration used for the calibration of reference compounds was 0.01−0.10 mg/mL. All standard and sample solutions were injected in triplicate.

Iron(III) to Iron(II) Reducing Activity. The ability of the extracts to reduce iron(III) was assessed by the method of Oyaizu (*22*). A 1 mL aliquot of each extract dissolved in water was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of a 1% aqueous (w/v) potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min incubation at 50 °C, 2.5 mL of 10% aqueous (w/v) trichloroacetic acid (TCA) was added, and the mixture was centrifuged for 10 min. A 2.5 mL aliquot of the upper layer was mixed with 2.5 mL of water and 0.5 mL of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. The mean absorbance values were plotted against concentration, and a linear regression analysis was carried out. The data are presented as ascorbic acid equivalents (AscAE) [mmol of ascorbic acid/g (dry wt) of sample], calculated from an ascorbic acid standard curve. The higher the AscAE value, the more potent is the iron(III) reductive power of the sample.

Iron(II) Chelation Activity. The chelation of iron(II) ions by the different extracts was carried out as described by Carter (*23*). To 200 μL of each extract were added 100 μL of 2.0 mM aqueous $FeCl_2 \cdot 4H_2O$ and 900 μL of methanol. The controls contained all the reaction reagents except the extract or positive control substance. After a 5 min incubation, the reaction was initiated by 400 μL of 5.0 mM ferrozine. After a 10 min equilibrium period, the absorbance at 562 nm was recorded. The iron(II)-chelating activity was calculated using eq 1, and

$$\%\ \text{inhibition} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \qquad (1)$$

the IC_{50} values were estimated by a nonlinear regression algorithm (SigmaPlot 2004 version 9.01). EDTA was used as a positive control.

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radical Scavenging. The ability of the extracts to scavenge DPPH• free radicals was determined by the method of Gyamfi et al. (*24*). A 50 μL aliquot of each extract, in Tris-HCl buffer (50 mM, pH 7.4), was mixed with 450 μL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH• in methanol. The controls contained all the reaction reagents except the extract or positive control substance. After a 30 min incubation in darkness and at ambient temperature (23 °C), the resultant absorbance was recorded at 517 nm. The percentage inhibition values were calculated using eq 1, and the IC_{50} values were estimated by a nonlinear regression algorithm (SigmaPlot 2004 version 9.01). Trolox, ascorbic and gallic acids, were used as positive controls.

Ascorbate−Iron(III)-Catalyzed Phospholipid Peroxidation. The ability of the extracts to scavenge hydroxyl radicals was determined by the method of Aruoma et al. (*25*). Bovine brain extract (Folch type VII) was mixed with 10 mM phosphate-buffered saline (PBS, pH 7.4) and sonicated in an ice bath until an opalescent suspension was obtained, containing 5 mg/mL phospholipid liposomes. The liposomes (0.2 mL) were combined with 0.5 mL of PBS buffer, 0.1 mL of 1 mM $FeCl_3$, and 0.1 mL of extract dissolved in PBS. Peroxidation was initiated by adding 0.1 mL of 1 mM ascorbic acid. The mixture was incubated at 37 °C for 60 min, after which 50 μL of 2.0% (w/v) butylated hydroxytoluene (BHT) was added to each tube, followed by 1 mL of 2.8% (w/v) TCA and 1 mL of 1.0% (w/v) 2-thiobarbituric acid (TBA). The samples were vortexed and heated in a water bath at 100 °C for 20 min. The reaction was stopped by placing the test tubes in an ice-H_2O bath for a 5 min period. To each tube was added 2 mL of *n*-butanol, and the mixture was vigorously vortexed. After centrifugation, the extent of oxidation was estimated from the absorbance of the organic layer at 532 nm. The percentage inhibition was calculated using eq 1, where the controls contain all the reaction reagents except the extract or positive control substance, and the IC_{50} values were estimated using a nonlinear regression algorithm (SigmaPlot 2004 version 9.01).

Non-Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation. The ability of the extracts to inhibit nonsite-specific hydroxyl radical-mediated peroxidation was carried out essentially as described by Halliwell et al. (*26*). The reaction mixture contained 500 μL of extract dissolved in KH_2PO_4−KOH buffer (50 mM, pH 7.4), 100 μL of 28 mM 2-deoxy-D-ribose in KH_2PO_4−KOH buffer, 200 μL of a premixed 100 μM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 μL of 1.0 mM H_2O_2, and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 37 °C for 60 min. Thereafter, 50 μL of 2.0% (w/v) BHT in ethanol was added to each tube followed by 1 mL of 2.8% (w/v) TCA and 1 mL of 1.0% (w/v) TBA in 0.05 M NaOH. The samples were vortexed and heated in a water bath at 100 °C for 20 min. The reaction was stopped by placing

Table 1. Extract Yield, Total Phenols, and Qualitative–Quantitative HPLC Data for the Commercial *E. angustifolium* Extracts

		identified components[a]							
sample[b]	EY[c]	TP[d]	L-ascorbic acid	gallic acid	protocatechuic acid	hyperoside	ellagic acid	octyl gallate	Σ
1	203.2	156.76 ± 11.42	17.66 ± 0.88	4.46 ± 0.16	nd[e]	3.53 ± 0.15	1.18 ± 0.06	0.08 ± 0.01	26.91 ± 0.91
2	189.9	194.44 ± 7.86	nd	9.34 ± 0.35	2.88 ± 0.14	2.93 ± 0.14	0.96 ± 0.05	0.12 ± 0.01	16.23 ± 0.40
3	193.6	145.51 ± 14.21	nd	6.44 ± 0.28	3.22 ± 0.15	3.00 ± 0.14	0.59 ± 0.02	0.15 ± 0.01	13.40 ± 0.35
4	350.0	209.41 ± 4.21	6.11 ± 0.31	4.47 ± 0.24	nd	2.92 ± 0.14	1.06 ± 0.05	0.09 ± 0.01	14.65 ± 0.42
5	284.0	206.94 ± 19.37	15.92 ± 0.80	4.70 ± 0.18	nd	2.82 ± 0.10	1.07 ± 0.06	0.08 ± 0.01	24.59 ± 0.83

[a] Values (mg/g) are expressed as means ± standard deviation ($n = 3$). [b] Samples: 1, Diod; 2, Express-service; 3, Lotos; 4, nonfermented; and 5, Tver. [c] EY: extract yield expressed as mg extract/g (dry wt) of plant material. [d] TP: total phenol content expressed as gallic acid equivalents, mg gallic acid/g (dry wt) of extract. [e] nd: not detected.

the test tubes in a ice−H_2O bath for a 5 min period. To each tube was added 2 mL of *n*-butanol, and the mixture was vigorously vortexed. After centrifugation, the extent of oxidation was estimated from the absorbance of the organic layer at 532 nm. The percentage inhibition was calculated using eq 1, where the controls contained all the reaction reagents except the extract or positive control substance, and the IC_{50} values were estimated using a nonlinear regression algorithm (SigmaPlot 2004 version 9.01).

Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation. The ability of the extracts to inhibit site-specific hydroxyl radical-mediated peroxidation was carried out as described in the non-site-specific hydroxyl radical-mediated peroxidation inhibition procedure, except that EDTA was replaced by buffer (*26*).

Statistical Analysis. Data were presented as mean values ± standard deviation. Analysis of variance was performed using the one way ANOVA procedure. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Extraction Yield and Total Phenol Content. The extract yields and total phenol content data for the water-soluble extracts are shown in **Table 1**. The extract yields ranged from 189.9 mg/g (Express-service) to 350.0 mg/g (nonfermented), increasing in the order Express-service < Lotos < Diod < Tver < nonfermented. No significant association between the amount of extractable components and the phenolic content as determined by the Folin−Ciocalteu ($r^2 = 0.533$, $p = 0.161$) and HPLC ($r^2 = 0.047$, $p < 0.725$) methods existed. Nor did there exist an association with iron(II) chelation ($r^2 = 0.014$, $p = 0.850$), DPPH• scavenging ($r^2 = 0.636$, $p = 0.106$), hydroxyl radical scavenging ($r^2 = 0.683$, $p = 0.084$), or site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.665$, $p = 0.092$) indices. A strong association did exist between extract yield and the AscAE ($r^2 = 0.791$, $p < 0.05$) and non-site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.948$, $p < 0.01$) indices.

The total phenol content estimated by the Folin−Ciocalteu method, expressed as milligrams of gallic acid per gram of extract, ranged from 145.51 ± 14.21 mg of gallic acid/g (Lotos) to 209.41 ± 4.21 mg of gallic acid/g (nonfermented), increasing in the order Lotos < Diod < Express-service < Tver < nonfermented (**Table 1**). No association was identified between the Folin−Ciocalteu total phenols and the HPLC total phenols ($r^2 = 0.010$, $p < 0.873$) or with iron(II) chelation ($r^2 = 0.119$, $p = 0.569$), hydroxyl radical scavenging ($r^2 = 0.503$, $p = 0.180$), and non-site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.325$, $p = 0.316$) indices. A significant association did exist between the Folin−Ciocalteu values and the AscAE ($r^2 = 0.900$, $p = 0.014$), DPPH• scavenging ($r^2 = 0.9162$, $p = 0.011$) and site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.989$, $p < 0.01$) indices.

The total phenolic content:total extractable components ratio ranged from 59.8% (nonfermented extract) to 102.4% (Express-service extract), suggesting that 40.2% of the nonfermented extract's content is of non-Folin−Ciocalteu-reactive substances, while all the components within the Express-service extract are redox-active, i.e., are Folin−Ciocalteu-reactive substances (*27*). The ratios for the Diod, Lotos, and Tver extracts were approximately equivalent at 77.2%, 75.2%, and 72.9%, respectively.

Qualitative−Quantitative HPLC Compositional Analysis. The data from the qualitative−quantitative analysis of the extracts made using high performance liquid chromatography coupled with photodiode array detection is presented in **Table 1**, while the chromatograms with detector responses at 280 and 360 nm overlaid are presented in **Figure 1**. The components L-ascorbic acid, gallic acid, protocatechuic acid, hyperoside (quercetin-3-D-galactoside), ellagic acid, kaempferol-3-*O*-glucoside, and octyl gallate (**Figure 2**) were identified by comparisons to the retention time and UV spectra of authentic standards analyzed under identical analytical conditions, while the quantitative data were calculated from their respective calibration curves. A number of components within the extracts could only be tentatively identified by chemical class from their chromatographic behavior and UV spectra. Accordingly, all the extracts contained relatively polar compounds (i.e. hydroxybenzoates and flavonoids), with levels of gallic acid, hyperoside, ellagic acid, and octyl gallate ranging from 4.46 ± 0.16 to 9.34 ± 0.35 mg/g, 2.82 ± 0.10 to 3.53 ± 0.15 mg/g, 0.59 ± 0.02 to 1.18 ± 0.06 mg/g, and 0.08 ± 0.01 to 0.15 ± 0.01 mg/g, respectively (**Table 1**). Ascorbic acid was identified in the Diod (17.66 ± 0.88 mg/g), nonfermented (6.11 ± 0.31 mg/g), and Tver (15.92 ± 0.80 mg/g) extracts, while protocatechuic acid was only identified in the Express-service (2.88 ± 0.14 mg/g) and Lotos (3.22 ± 0.15 mg/g) extracts (**Table 1**).

There appeared to be no significant association between the HPLC-determined phenol content and the AscAE ($r^2 = 0.003$, $p < 0.934$), iron(II) chelation ($r^2 = 0.494$, $p < 0.186$), DPPH• scavenging ($r^2 = 0.042$, $p < 0.741$), hydroxyl radical scavenging ($r^2 = 0.477$, $p = 0.197$), and non-site- ($r^2 = 0.059$, $p = 0.694$) and site-specific ($r^2 = 0.032$, $p = 0.772$) 2-deoxy-D-ribose degradation indices.

Iron(III) to Iron(II) Reducing Activity. The antioxidant activity of an extract is considered to be related to its reductive activity (*28*, *29*). Thus, to assess the electron-donating properties of the extracts, their ability to reduce iron(III) was assessed. In **Figure 3**A, it can be seen that the extracts possessed the ability to reduce iron(III) and did so in a linear concentration-dependent fashion (data not shown). On the basis of the AscAE values, expressed as millimoles of ascorbic acid/gram of extract

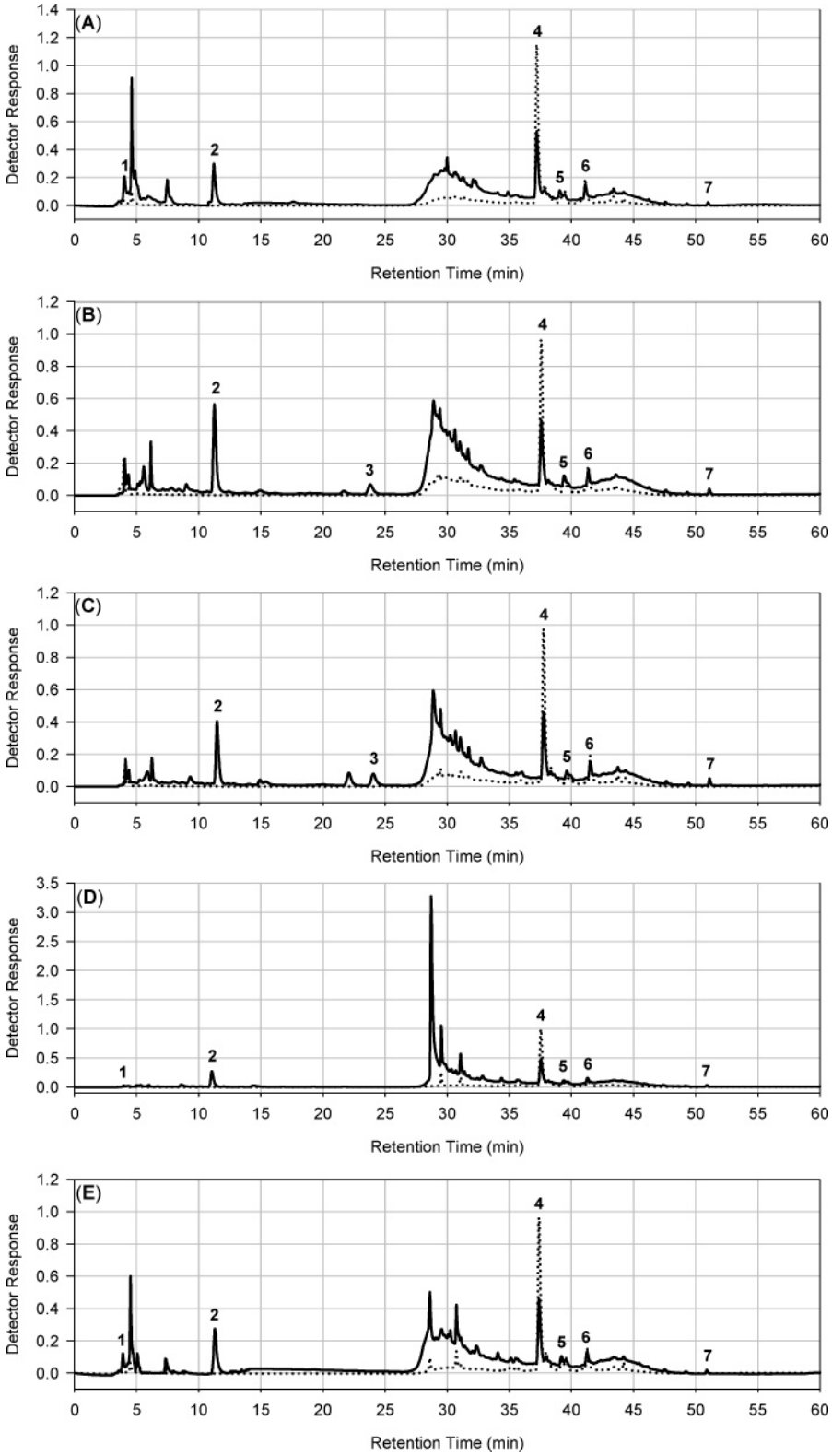

Figure 1. HPLC–PDA analysis of the water-soluble (**A**) Diod, (**B**) Express-service, (**C**) Lotos, (**D**) nonfermented, and (**E**) Tver *E. angustifolium* extracts with detector responses at 280 (straight line) and 360 nm (dotted line) overlaid. **1**, L-Ascorbic acid; **2**, gallic acid; **3**, protocatechuic acid; **4**, hyperoside; **5**, ellagic acid; **6**, kaempferol-3-*O*-glucoside; and **7**, octyl gallate.

calculated from the plots of absorbance versus sample concentration, the nonfermented extract was the most effective iron-(III) reducer, followed by the Tver extract, which was statistically indistinguishable ($p > 0.05$) from the Express-service extract. It was not possible to distinguish between the efficacies of the Diod, Express-service, and Lotos extracts, which were indistinguishable ($p > 0.05$) from the Express-service extract (**Figure 3A**). Despite the ability of extracts to reduce iron(III), none were equivalent to either ascorbic acid or gallic acid in this assay. There was a significant association between the

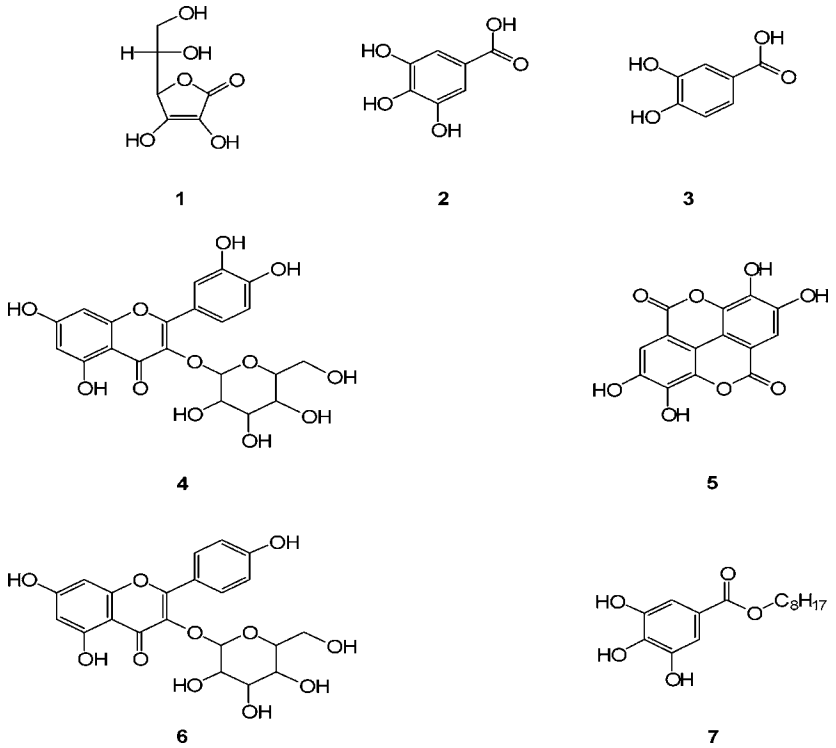

Figure 2. Structural formulas of the identified components within the commercial *E. angustifolium* extracts: **1**, L-ascorbic acid; **2**, gallic acid; **3**, protocatechuic acid; **4**, hyperoside; **5**, ellagic acid; **6**, kaempferol-3-*O*-glucoside; and **7**, octyl gallate.

AscAE index and DPPH• scavenging ($r^2 = 0.852$, $p = 0.025$) and site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.944$, $p < 0.01$) indices; however, no apparent association existed between the AscAE index and the iron(II) chelation ($r^2 = 0.056$, $p = 0.703$), hydroxyl radical scavenging ($r^2 = 0.561$, $p = 0.145$), and non-site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.610$, $p = 0.119$) indices.

The iron(III) reduction data suggests that the extracts are capable of participating in electron-transfer reactions and, therefore, should be able to donate electrons to reactive free radicals, converting them into more stable nonreactive products.

Iron(II) Chelation. An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydroperoxide decomposition and Fenton-type reactions (*30*). Therefore, it was considered of importance to screen the iron(II)-chelating ability of the extracts. All the extracts demonstrated the ability to chelate iron(II) ions (**Figure 3B**) and did so in a concentration-dependent fashion (data not shown). On the basis of the estimated IC_{50} values, the concentration of extract required to chelate 50% of the available iron(II) species as estimated using a nonlinear regression algorithm, the Lotos extract was the most effective iron(II) chelator. The Diod, Express-service, nonfermented, and Tver extracts were less effective and were statistically ($p > 0.05$) indistinguishable. Despite the iron(II)-chelating activity of the extracts, none were as effective as the positive control, EDTA. There was no significant association between iron(II) chelation and DPPH• ($r^2 = 0.052$, $p = 0.711$) and hydroxyl radical ($r^2 = 0.331$, $p = 0.310$) scavenging and non-site- ($r^2 = 0.01$, $p = 0.871$) and site-specific ($r^2 = 0.124$, $p = 0.561$) 2-deoxy-D-ribose degradation indices. This suggests that there is no common underpinning mechanism of action between iron(II) chelation and these redox-dependent indices.

From the iron(II) chelation data, the extracts maybe able to play a protective role against oxidative damage by sequestering iron(II) ions that may otherwise catalyze Fenton-type reactions or participate in metal-catalyzed hydroperoxide decomposition reactions (*30*).

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radical Scavenging. A cardinal property of an antioxidant is the ability to scavenge free radicals. Free radicals are involved in the process of lipid peroxidation, are considered to play a fundamental role in several chronic diseases, such as cancer and cardiovascular disease among others, and are implicated in the aging process. Therefore, it was considered important to assess the free radical scavenging efficacy of the extracts. This was estimated using the synthetic nitrogen-centered species DPPH•. This free radical serves as both the oxidizable substrate, which can be reduced by an electron- or hydrogen-donating compound to its hydrazine derivative, and as the reaction indicator molecule (*31*).

All the extracts were capable of scavenging DPPH• radicals (**Figure 3C**) and did so in a concentration-dependent fashion (data not shown). From the estimated IC_{50} values, it can be seen that the increasing order of activity was Tver (which was not statistically distinguishable ($p > 0.05$) from nonfermented) > Express-service (which was statistically indistinguishable ($p > 0.05$) from nonfermented) > Diod and Lotos extracts, which were statistically indistinguishable ($p > 0.05$). The DPPH• scavenging activity of the Tver extract was as effective as that of the water-soluble α-tocopherol analogue Trolox but was not as effective as either ascorbic acid or gallic acid. A strong association between DPPH• scavenging and the AscAE ($r^2 = 0.852$, $p < 0.025$) and site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.944$, $p < 0.01$) indices was observed; however, no such relationship appeared to occur with the iron(II) chelation ($r^2 = 0.053$, $p = 0.711$), hydroxyl radical scavenging ($r^2 = 0.603$, $p = 0.122$), and non-site-specific ($r^2 = 0.411$, $p = 0.224$) indices.

The DPPH• scavenging data suggests that the extracts are capable of scavenging free radicals at physiological pH; thus, they should be able to prevent the initiation and propagation of free radical-mediated chain reactions by stabilizing reactive

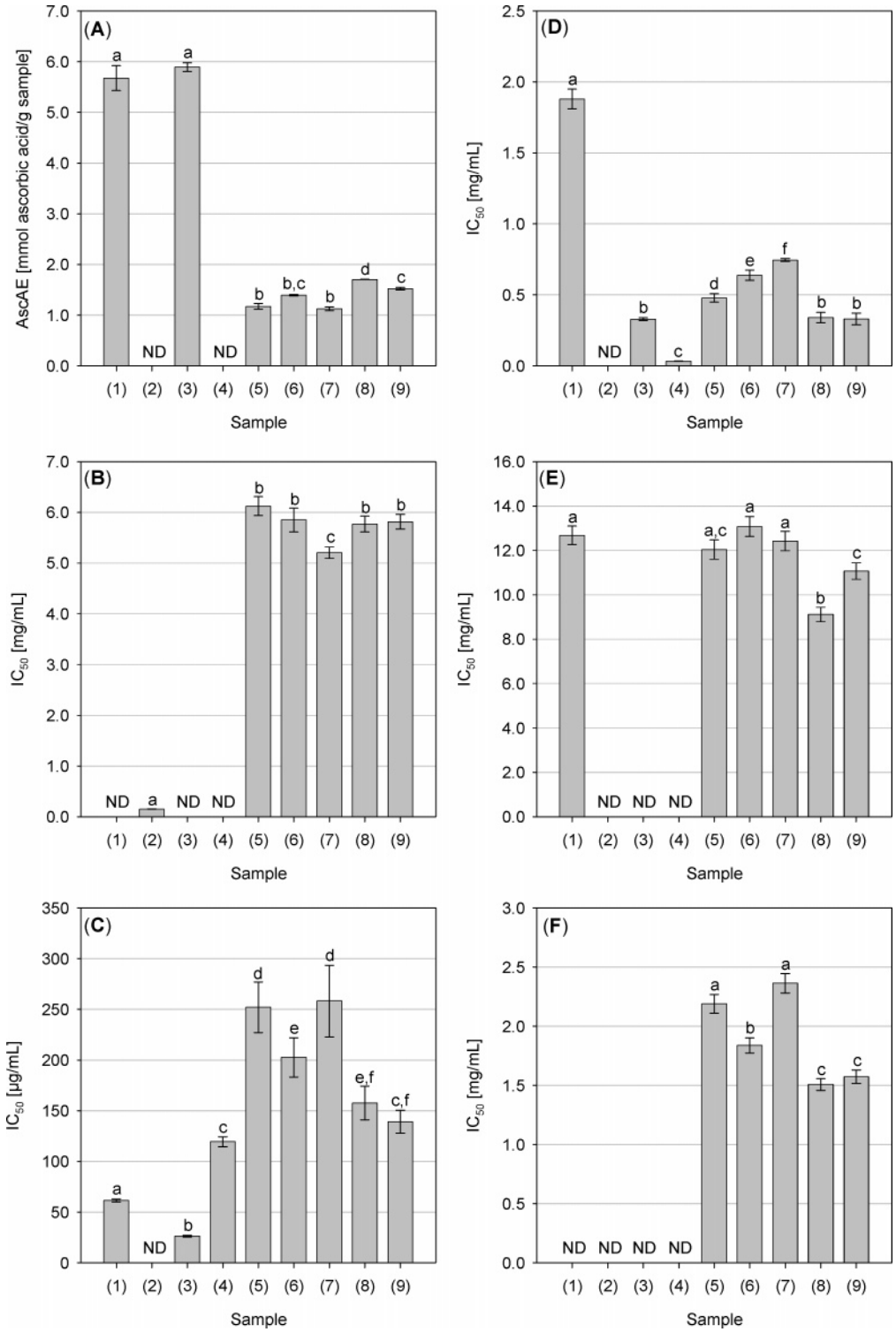

Figure 3. The effect of the extracts and positive controls upon (**A**) iron(III) reduction, (**B**) iron(II) chelation, (**C**) DPPH• radical scavenging, (**D**) ascorbate-iron(III)-catalyzed phospholipid peroxidation, and (**E**) non-site- and (**F**) site-specific hydroxyl radical-mediated 2-deoxy-d-ribose degradation. Values are presented as means ± standard deviation. Bars with the same lowercase letter (a–e) are not significantly ($p > 0.05$) different. Samples: 1, ascorbic acid; 2, EDTA; 3, gallic acid; 4, Trolox; 5, Diod; 6, Express-service; 7, Lotos; 8, nonfermented; and 9, Tver. ND, not determined.

species via electron- or hydrogen-donation before such deleterious reactions can occur.

Ascorbate−Iron(III)-Catalyzed Phospholipid Peroxidation. The iron(III) reduction and DPPH• scavenging models are potential indicators of antioxidant activity; however, neither method utilizes a food or biologically relevant oxidizable substrate, so no direct information on an extract's protective properties can be ascertained. Furthermore, although an antioxidant may effectively reduce reactive free radicals, it may not be able to efficiently reduce iron(III) and vice versa (*32*). Thus, it was considered important to assess the free radical scavenging ability of the extracts with a more relevant matrix (i.e. bovine brain-derived phospholipids) and reactive oxygen species (i.e. the hydroxyl radical).

Phospholipid liposomes rapidly undergo peroxidation in the presence of iron(III) and a reducing agent at 37 °C (*25*),

producing malonaldehyde and associated aldehydes (*33*). These byproducts are 2-thiobarbituric acid reactive species (TBARS) with an absorption maximum of 532 nm. By measuring the absorbance at this wavelength, it is possible to estimate the hydroxyl radical scavenging properties of an extract and its effect upon lipid peroxidation.

All the extracts were able to inhibit the formation of phospholipid-derived TBARS by scavenging hydroxyl radicals generated by ascorbate−iron(III)-catalyzed Fenton chemistry (**Figure 3D**) and did so in a concentration-dependent fashion (data not shown). On the basis of the estimated IC_{50} values, the hierarchy of hydroxyl radical scavenging was nonfermented and Tver extracts (statistically ($p > 0.05$) indistinguishable) > Diod > Express-service > Lotos. The hydroxyl radical scavenging activity of the nonfermented and Tver extracts was indistinguishable from that of gallic acid, while all the extracts were more potent than ascorbic acid, which was poorly active in this model system. Only the positive control Trolox was a much better hydroxyl radical scavenger. There was no association between hydroxyl radical scavenging index and the non-site- ($r^2 = 0.616$, $p = 0.116$) and site-specific ($r^2 = 0.627$, $p = 0.110$) 2-deoxy-D-ribose degradation indices.

According to the hydroxyl radical scavenging data, the extracts were capable of affording protection to phospholipids against oxidative degradation mediated by hydroxyl radicals, an extremely reactive oxygen species capable of interacting with every biologically relevant molecule within its vicinity. Thus, the extracts should be able to protect susceptible lipid-rich substrates, e.g. foodstuffs and biological membranes, from oxidative degradation.

Non-Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation. When EDTA-chelated iron(III) ions are incubated at physiological pH with a reducing agent and H_2O_2, hydroxyl radicals are generated. These hydroxyl radicals are capable of migrating to a susceptible substrate and fragmenting it into TBARS. This chemistry is used in the non-site-specific hydroxyl radical-mediated 2-deoxy-D-ribose assay. An extract that inhibits the formation of TBARS in this system can be considered to contain components capable of scavenging hydroxyl radicals and protecting carbohydrates from oxidative degradation (*34*).

All the extracts were capable of inhibiting the formation of 2-deoxy-D-ribose-derived TBARS (**Figure 3E**) and did so in a concentration-dependent fashion (data not shown). On the basis of the estimated IC_{50} values, the extracts could be grouped into three categories: (i) highly active, a group consisting of the nonfermented extract; (ii) moderately active, a group consisting of the Tver extract, and (iii) poorly active, a group containing the Diod, Express-service, and Lotos extracts (all of which were significantly ($p > 0.05$) indistinguishable). The nonfermented and Tver extracts were significantly better scavengers of hydroxyl radicals in this model system than ascorbic acid, while the Diod, Express-service, and Lotos were as effective as this positive control substance. No significant association existed between the non-site-specific and the site-specific 2-deoxy-D-ribose degradation ($r^2 = 0.457$, $p = 0.211$) indices.

The extracts were capable of preventing the hydroxyl radical-mediated degradation of 2-deoxy-D-ribose into TBARS at physiological pH. Therefore, the extracts contain components that may be able to protect carbohydrate components in vivo and in foods, cosmetics, and pharmaceutical preparations from oxidative damage.

Site-Specific Hydroxyl Radical-Mediated 2-Deoxy-D-ribose Degradation. In the site-specific hydroxyl radical-mediated 2-deoxy-D-ribose assay, iron(III) binds 2-deoxy-D-ribose, and hydroxyl radicals are generated at the surface of the carbohydrate molecule, which then fragment it into TBARS. It is implausible that it is possible to intercept these hydroxyl radicals before fragmentation of the 2-deoxy-D-ribose occurs. A more conceivable scenario is that an extract that inhibits the formation of TBARS in this system does not scavenge hydroxyl radicals but prevents their formation by chelating−deactivating iron(III) (*34*). Thus, it was considered necessary to repeat the aforementioned experiment without EDTA. Furthermore, it has been reported that the 2-deoxy-D-ribose assay when performed with EDTA is rather artificial when extrapolated to in vivo activity (*34*).

All the extracts were capable of inhibiting the formation TBARS (**Figure 3F**) and did so in a concentration-dependent fashion (data not shown). On the basis of the estimated IC_{50} values, the extracts could be categorized as (i) highly active, a group consisting of the nonfermented and Tver extracts; (ii) moderately active, a group consisting of the Express-service extract, and (iii) poorly active, a group consisting of the Diod and Lotos extracts (**Figure 3F**).

The extracts were capable of preventing hydroxyl radical-mediated oxidative degradation of 2-deoxy-D-ribose at physiological pH by interrupting the generation of hydroxyl radicals via a mechanism of iron(III) chelation and deactivation. Thus, the extracts may be able to prevent oxidative degradation of susceptible products, e.g. foodstuffs, cosmetics, and pharmaceutical preparations.

Five commercial water-soluble extracts of *E. angustifolium* L. (Onagraceae), commonly known as willow herb, were screened in a battery of in vitro antioxidant assays. Furthermore, the Folin−Ciocalteu phenol content and qualitative-quantitative analysis of the analytes within each sample were determined. The qualitative−quantitative analysis revealed that the extracts principally contained polar compounds present in varying amounts, viz., hydroxylated benzoic acids and flavonoids. Identified components included ascorbic acid, gallic acid, protocatechuic acid, ellagic acid, octyl gallate, hyperoside, and kaempferol-3-*O*-glucoside. Two extracts (nonfermented and Tver) consistently out performed the remaining extracts in the antioxidant screening assays, except in the case of iron(II) chelation, where the Lotos extract was statistically ($p < 0.05$) better. This trend could be explained by the observation that both of the nonfermented and Tver extracts contained the highest total phenol content, as estimated by the Folin−Ciocalteu reagent method.

Overall, one may conclude from the data that the *E. angustifolium* extracts, and in particular the nonfermented and Tver water-soluble extracts, possess exploitable in vitro antioxidant properties. The potency of the two most active extracts may be explained by their relatively high content of phenolic substances, as determined by the Folin-Ciocalteu reagent method; however, due to the often less than significant correlations phenolic content and activity, it is clear that having a high phenolic content per se does not necessarily result in a high degree of activity in all model systems. As to potential applications for these extracts, they may have a role in the functionalization of foods or as ingredients for nutraceuticals. However, based solely upon the presented data, one cannot make this assumption with confidence. Further research should be carried out to determine (i) whether the components within the extracts are absorbed through the gastrointestinal tract, (ii) whether they possess demonstrable in vivo antioxidant properties, and (iii) what are their acute and chronic tolerable doses.

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