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Antioxidant Activities of Extracts from *Barkleyanthus salicifolius* (Asteraceae) and *Penstemon gentianoides* (Scrophulariaceae)†

MARIANA DOMÍNGUEZ,‡ ANTONIO NIETO,‡ JUAN C. MARIN,‡ ANNA-SIGRID KECK,# ELIZABETH JEFFERY,*,# AND CARLOS L. CÉSPEDES*,‡

Chemical Ecology Lab.2-C, Instituto de Quimica, Ciudad Universitaria, Coyoacán 04510, Mexico D.F., Mexico, and Department of Food Science and Human Nutrition, 499 Bevier Hall, U niversity of Illinois, 905 South Goodwin Avenue, Urbana, Illinois 61801

Various extracts of the aerial parts of *Barkleyanthus salicifolius* (Asteraceae) and *Penstemon gentianoides* (Scrophulariaceae) have been used in folk medicine to treat many ailments, particularly inflammation and migraine. Neither the bioactive components responsible nor the mechanisms involved have been evaluated. Here are reported antioxidant activities of their methanol, dichloromethane, and ethyl acetate extracts. Samples were evaluated for oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging, and inhibition of the formation of thiobarbituric acid reactive species (TBARS), a measure of lipid peroxidation. Antioxidant activities were strongly correlated with total polyphenol content. The most active extracts from *P. gentianoides* in scavenging DPPH radicals and inhibiting TBARS formation were the methanol extract (A) and a further ethyl acetate extract of this (E). Partition E was further divided into eight fractions, and both E and the fractions were compared for activity against butylated hydroxytoluene, quercetin, and tocopherol. Partition E and the most active fractions, 5 and 6, were found to have I_{50} values of 14.1, 38.6, and 41.8 ppm, respectively, against DPPH and 18.5, 26.0, and 12.7 ppm, respectively, against TBARS formation. Consistent with this finding, partition E and fractions 4−6 had the greatest ORAC and FRAP values. These results show that these plants could be useful antioxidant sources.

KEYWORDS: Flavonoids; phenols; antioxidant activity; *Barkleyanthus*; Asteraceae; *Penstemon*; Scrophulariaceae

INTRODUCTION

Antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (*1*). This concept is fundamental to food chemistry, in which synthetic antioxidants such as butylated hydroxytoluene (BHT) have long been used to preserve the quality of food by protecting against oxidation-related deterioration. A growing body of literature points to the importance of natural antioxidants from many plants that may be used to reduce oxidative damage, not only in foods but also in the human body. This may provide protection against chronic diseases, including cancer and neurodegenerative diseases, inflammation, and cardiovascular disease. Adverse conditions within the environment, such as smog and UV radiation, in addition to diets rich in saturated fatty acids, increase oxidative damage in the body. Given this constant exposure to oxidants, antioxidants may be necessary to counteract chronic oxidative effects, thereby improving the quality of life (*2*).

Increasing interest in the measurement of the antioxidant activity of different plant samples is derived from the overwhelming evidence of the importance of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$), and nitric oxide ($NO^{\bullet}$) radicals in aging and chronic disease (*3*). Several methods have been developed to measure the antioxidant activity in biological samples, including the oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and inhibition of formation of thiobarbituric acid reactive species (TBARS) (*4*).

The use of traditional medicine is widespread, and plants still present a large source of novel active biological compounds with different activities, including anti-inflammatory, anticancer, antiviral, and antibacterial activities. Antioxidants may play a role in these health-promoting activities (*5*). In the continuation of our general screening program of Mexican flora with

* Authors to whom correspondence should be addressed [(C.L.C.) telephone +52-55-5622-4447, fax +52-55-5616-2203, e-mail ccespede@servidor.unam.mx; (E.J.) telephone (217) 333-3820, e-mail ejeffery@uiuc.edu].

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‡ Universidad Nacional Autónoma de México.

University of Illinois.

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biological activities (*6*), an examination of the extracts of *Penstemon gentianoides* (Scrophulariaceae) and *Barkleyanthus salicifolius* (Asteraceae) has been initiated. *P. gentianoides* (HBK) Poiret, Lindl. Don. Scrophulariaceae (common names: beardtongue, "jarritos", and "jarra") grows in hilly areas above 3000 m in forest communities dominated by *Abies religiosa*, *Alnus jorullensis*, *Pinus pseudostrobus*, and *Pinus hartwegii*. The presence of *P. gentianoides*, as with *Senecio cinerarioides*, *Senecio salignus*, *Muhlenbergia macorura*, *Festuca tolucensis*, and *Lupinus montanus*, has been used to indicate environmental disturbance (*7*). There are no previous ethnobotanical or phytochemical studies of this plant, although it has enjoyed popularity as an ethnomedicine for many years, used particularly as an anti-inflammatory agent.

Barkleyanthus salicifolius (Kunth) H. Rob & Brettell (Ex *Senecio salignus*) Asteraceae (common names: willow ragwort, "Jara", "jaktin", "jara-tokstini", "jaralillo", and "jarilla") is a common shrub ~1.5 m high, growing alone or in association with *Poaceae* spp. on disturbed land between pine forests on hillsides at 1200−3000 m throughout northern Mexico and into New Mexico, Texas, and Arizona (*8*). This plant also enjoys popular use as an anti-inflammatory, particularly for protection against rheumatism, migraines, and liver and kidney disease.

There are no reported studies relating these properties to any bioactivity or to any chemical composition such as phenolic content of the extracts of these medicinal plants. The present investigation evaluates the antioxidant activity of these two plants, *P. gentianoides* and *B. salicifolius*, and its relationship to the presence of phenolic compounds in these plants.

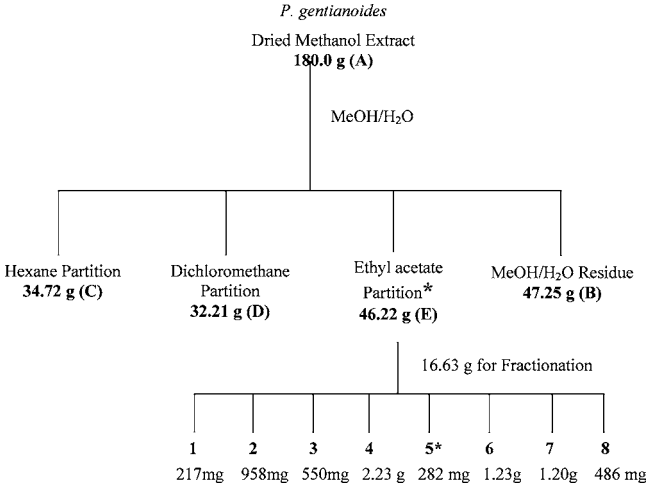
MATERIALS AND METHODS

Biological Material. *B. salicifolius* (Kunth) H. Rob & Brettell (Ex *S. salignus*) Asteraceae-Compositae was collected from the roadside between Mexico City and Oaxtepec, at Juchitepec, in March 2002. *P. gentianoides* (HBK) Poiret, Lindl. Don. Scrophulariaceae was collected on the highest hills (>3000 m) within the Park "Los Dinamos", near Mexico City, in October 2002. The plants were identified botanically by Professor Francisco Ramos (Instituto de Biologia, UNAM), and voucher specimens were deposited at the Herbarium of the Biology Institute at UNAM (MEXU). The collected plants were air-dried and prepared for extraction. From both species were separated the main morphological parts (flowers, stems, and leaves), which were then were milled and extracted with methanol; six extracts were obtained, which were then partitioned with *n*-hexane, dichloromethane, and ethyl acetate. Furthermore, the ethyl acetate partition of *P. gentianoides* was fractionated into eight subfractions (**Scheme 1**).

The aerial parts of *P. gentianoides* were separated into their morphological parts (stems, leaves, and flowers), before being dried and milled. On the basis of their popular use as ethnomedicines, only samples of leaves and flowers were studied. Leaf samples were extracted into hexane, dichloromethane, ethyl acetate, or methanol. Because most of the activity was associated with the methanol extract (data not shown), only this extract was evaluated further. The methanol extract of the leaves (A) was dried and redissolved in methanol/water (6:4) and then partitioned into hexane (C), dichloromethane (D), and ethyl acetate (E), leaving a residue (B), as shown in **Scheme 1**. The ethyl acetate partition (E) showed high antioxidant activity and was further fractionated into eight fractions (1−8), by open column chromatography using silica gel (type G, 10−40 μm, Sigma-Aldrich) as solid phase. Elution was carried out with hexane/ethyl acetate, then with ethyl acetate/methanol mixtures with increasing gradient of polarity, and finally with 100% methanol; all fractions were analyzed by TLC as antioxidant bioautographic assay (*19*, *20*) using different solvent systems (hexane/ethyl acetate and dichloromethane/methanol mixtures).

Due to the small quantity of material, flowers of *P. gentianoides* were extracted into only methanol (F). Compared to the methanol extracts of *P. gentianoides*, the *B. salicifolius* methanol extracts from leaves (G) and flowers (H) were less effective at inhibiting the reduction of the DPPH free radical or at inhibiting TBARS formation (**Table 1**). For this reason these extracts were not partitioned further. The above-mentioned extracts and partitions (A−H) were submitted to a number of analyses, including ORAC, FRAP, DPPH, and TBARS, and were evaluated for total phenolic content using the Folin−Ciocalteu method (*12*, *13*).

Scheme 1. Method of Obtaining Extracts, Partitions, and Fractions[a]



[a] Not shown in this scheme are extract F (flowers of *P. gentianoides*), extract G (leaves of *B. salicifolius*), and extract H (flowers of *B. salicifolius*).

Table 1. Amounts of *P. gentianoides* and *B. salicifolius* Extracts Needed To Inhibit Oxidative Damage by 50%

species	sample[a]	DPPH[b]	TBARS[c]
P. gentianoides	A	21.4 ± 1.9b	29.3 ± 2.1b
	B	19.9 ± 1.3b	27.0 ± 3.2b
	C	273.7 ± 17.9c	>1000
	D	47.9 ± 3.1a	42.4 ± 2.7c
	E	14.1 ± 1.3d	18.5 ± 1.2a
	F	30.6 ± 2.5e	nd
B. salicifolius	G	164.7 ± 12.9b	96.2 ± 4.1d
	H	300.7 ± 21.7b	44.8 ± 2.9c

[a] See **Scheme 1** for an explanation of extracts and partitions. [b] IC_{50} for inhibition of diphenylpicrylhydrazyl radical formation. Mean ± SD, $n = 3$. Different letters show significant differences at $P < 0.05$, using Duncan's multiple-range test. [c] IC_{50} for inhibition of peroxidation of lipids, estimated as thiobarbituric acid reactive substances. Values are expressed as μg/mL (ppm). See Materials and Methods for details. Mean ± SD, $n = 3$. Different letters show significant differences at $P < 0.05$, using Duncan's multiple-range test. nd, not determined.

Chemicals and Solvents. All reagents used were of either analytical grade or chromatographic grade. 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl [2,2-diphenyl-1-(2,4,6-trinitrophenyl **(**DPPH)], butylated hydroxytoluene (BHT; 2[3]-*tert*-butyl-4-hydroxytoluene), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), quercetin, Folin−Ciocalteu reagent, (+)-catechin, 2-thiobarbituric acid (TBA), 2,4,6-tripyridyl-*s*-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$, fluorescein disodium (FL), 3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9[9*H*]-xanthen]-3-one, tetramethoxypropane (TMP), Tris-HCl buffer, $FeSO_4$, and trichloroacetic acid were purchased from Sigma-Aldrich Quimica, S.A. de C.V., Toluca, Mexico, or Sigma, St. Louis, MO. Methanol, CH_2Cl_2, $CHCl_3$, NaCl, KCl, KH_2PO_4, $NaHPO_4$, NaOH, KOH, HCl, sodium acetate trihydrate, glacial acetic acid, silica gel GF_{254} analytical chromatoplates, silica gel grade 60, (70−230, 60 Å) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck-Mexico, S.A.

Oxygen Radical Absorbance Capacity Estimation. ORAC measures the antioxidant scavenging activity of a sample or standard against peroxyl radicals generated from AAPH at 37 °C using FL; Trolox was used as standard (*9*, *10*). The assay was carried out in black-walled

96-well plates (Fischer Scientific, Hanover Park, IL), at 37 °C in 75 mM phosphate buffer (pH 7.4). The following reactants were added in the order shown: sample or Trolox (20 μL; 7 μM final concentration) and fluorescein (120 μL; 70 nM final concentration). The mixture was preincubated for 15 min at 37 °C, after which AAPH (60 μL; 12 mM final concentration) was added (final volume = 200 μL). The microtiter plate was immediately placed in a Biotek model FLx800 (Biotek Instruments, Inc., Winooski, VT) fluorescence plate reader set, and the fluorescence was recorded every minute for 120 min, using an excitation λ = 485/20 nm and en emission λ = 582/20 nm, to reach a 95% loss of fluorescence. Results are expressed as micromoles of Trolox equivalents (TE) per gram. All tests were conducted in triplicate.

Ferric Reducing Antioxidant Power Estimation. The FRAP assay was performed as previously described by Benzie and Strain (*11*). Reagents were freshly prepared and mixed in the proportion 10:1:1, for A/B/C, where A = 300 mM sodium acetate trihydrate/glacial acetic acid buffer (pH 3.6), B = 10 mM TPTZ in 40 mM HCl, and C = 20 mM $FeCl_3$. Catechin was used for a standard curve (5−40 μM final concentration) with all solutions, including samples, dissolved in sodium acetate trihydrate/glacial acetic acid buffer. The assay was carried out in 96-well plates, at 37 °C at pH 3.6, using 10 μL of sample or standard plus 95 μL of the mixture of regents shown above. After 10 min of incubation at room temperature, absorbance was read at 593 nm. Results are expressed as micromoles of catechin equivalents (Cat E) per gram of sample. All tests were conducted in triplicate.

Estimation of Total Polyphenol Content. The total phenolic content of extracts was determined using the Folin−Ciocalteu reagent (*12*, *13*): 10 μL of sample or standard (10−100 μM catechin) plus 150 μL of diluted Folin−Ciocalteu reagent (1:4 reagent/water) was placed in each well of a 96-well plate and incubated at room temperature for 3 min. Following the addition of 50 μL of sodium carbonate (2:3 saturated sodium carbonate/water) and a further incubation of 2 h at room temperature, absorbance was read at 725 nm. Results are expressed as micromoles of Cat E per gram. All tests were conducted in triplicate.

Estimation of Lipid Peroxidation. As an index of lipid peroxidation, TBARS levels were measured using rat brain homogenates according to the method described by Ng et al. (*14*), with some modifications. Adult male Wistar rats (200−250 g) were provided by the Instituto de Fisiología Celular, UNAM, and their use was approved by the Animal Care and Use Committee (PROJ.-NOM 087-ECOL-SSA 1-2000). Rats were maintained at 25 °C on a 12/12 h light/dark cycle with free access to food and water and sacrificed under mild ether anesthesia. Cerebral tissue was rapidly dissected from the whole brain and homogenized in phosphate-buffered saline (PBS; 0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 2.16 g of $NaHPO_4 \cdot 7\ H_2O$/L, pH 7.4) to produce a 1 in 10 homogenate (w/v) (*15*). The homogenate was centrifuged for 10 min at 3400 rpm, and the resulting pellet was discarded. The protein content of the supernatant was measured according to the method of Lowry (*16*), and samples were adjusted to 2.5 mg of protein/mL with PBS. The supernatant (400 μL, 1 mg of protein) was preincubated with sample (50 μL) at 37 °C for 30 min, then peroxidation was initiated by the addition of 50 μL of freshly prepared $FeSO_4$ solution (final concentration = 10 μM), and the sample was incubated at 37 °C for an additional 1 h (*14*). The TBARS assay was determined as described by Ohkawa et al. (*17*) with the modification that 0.5 mL of TBA reagent (1% thiobarbituric acid in 0.05 N NaOH and 30% trichloroacetic acid, 1:1) was used and that the final solution was cooled on ice for 10 min, centrifugated at 10000 rpm for 5 min, and then heated at 95 °C in a boiling water bath for 30 min. After cooling on ice, the absorbance was read at 532 nm in a Spectronic Genesys 5 spectrophotometer. Quercetin and BHT were used as positive controls. Concentrations of TBARS were calculated using a TMP standard curve (*18*). Results are expressed as nanomoles of TBARS per milligram of protein, with percent inhibition after 30 min calculated as the inhibition ratio (IR), where

$$\text{IR}\ (\%) = [(C - E)/C] \times 100$$

where C = absorbance of the control and E = absorbance of the test sample. These values were plotted against the log of the concentrations of individual extracts and fractions, and a decrease of 50% in peroxidation was defined as the EC_{50}.

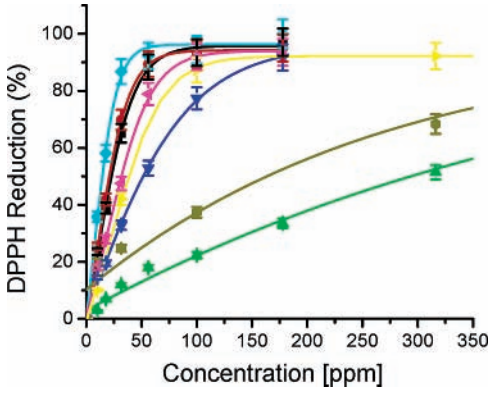


Figure 1. Scavenging of DPPH radicals by extracts from *P. gentianoides*. For explanation of the extracts and partitions, see **Scheme 1**. Extracts and partitions: G (olive green), H (green), D (dark blue), E (light blue), A (magenta), and F (yellow). Standard compounds: α-tocopherol (black) and quercetin (red).

Reduction of the 2,2-Diphenyl-1-Picrylhydrazyl Radical. Extracts and partitions were chromatographed on TLC and examined for antioxidant effects by spraying the TLC plates with DPPH reagent. Specifically, the plates were sprayed with 0.2% DPPH in methanol (*19*). Plates were examined 30 min after spraying, and active compounds appear as yellow spots against a purple background. In addition, TLC plates were sprayed with 0.05% β-carotene solution in chloroform and then held under UV_{254} light until the background bleached. Active components appeared as pale yellow spots against a white background (*20*). Samples that showed a strong response were selected for fractionation by open column chromatography, using solvents of increasing polarity. Furthermore, each fraction was analyzed with DPPH in microplates of 96 wells as follows: extracts, partitions, and fractions (50 μL) were added to 150 μL of DPPH (100 μM, final concentration) in methanol (the microtiter plate was immediately placed in a Biotek model ELx808) and their absorbances read at 515 nm after 30 min (*21*, *22*). Quercetin and α-tocopherol were used as standards.

Statistical Analysis. Data were analyzed by one-way ANOVA followed by Dunnett's test for comparisons against control. Values of $P \leq 0.05$ (*) and $P \leq 0.01$ (**) were considered to be statistically significant, and the significant differences between means were identified by GLM procedures. In addition, differences between treatment means were established with a Student−Newman−Keuls (SNK) test. The I_{50} values for each analysis were calculated by Probit analysis. Complete statistical analyses were performed using the MicroCal Origin 6.2 statistical and graphs PC program.

RESULTS AND DISCUSSION

The DPPH radical scavenging assay was used first as a screen for antioxidant components within the primary extracts (*19*, *20*). As shown in **Figure 1** and **Table 2**, the ethyl acetate partition (E) had the higher inhibitory activity against DPPH radical formation compared to the other partitions, with an I_{50} value of 14.1 ppm (**Table 1**). For extracts/partitions A, B, C, D, F, G, and H the I_{50} values were 21.4, 19.9, 273.7, 47.9, 30.6, 164.7, and 300.7 ppm, respectively. Almost all of these samples exhibited a concentration dependence in their DPPH radical scavenging activities, particularly E, which showed the highest activity (86.8% inhibition) at a concentration of 31.6 ppm (**Figure 1**). This action was greater than that of α-tocopherol, which at 31.6 ppm caused only 53.8% quenching (data not shown). Partition E was then loaded onto a silica gel open chromatography column, from which eight fractions were collected (1−8). Of these, fraction 5 was the most active, with an IC_{50} of 38.6 ppm (**Table 2**).

In addition to samples B, D, and F, fractions 3, 4, 5, and 6 of partition E showed considerable activity, quenching DPPH

Table 2. Amounts of Fractions from Ethyl Acetate Partition E of *P. gentianoides* Needed To Inhibit Oxidative Damage by 50%[a]

group	DPPH[b]	TBARS[c]
1	>1000	nd
2	57.4 ± 3.2a	30.0 ± 0.997a
3	45.3 ± 3.8b	20.1 ± 1.790b
4	45.2 ± 2.9b	23.6 ± 1.011b
5	38.6 ± 2.7b	26.0 ± 2.186b
6	41.8 ± 3.2b	12.7 ± 1.661c
7	52.3 ± 4.0a	30.3 ± 2.165a
8	65.8 ± 3.7c	28.2 ± 3.149a

[a] Values expressed as μg/mL (ppm). Mean ± SD, $n = 3$. Different letters show significant differences at $P < 0.05$, using Duncan's multiple-range test. [b] IC_{50} for inhibition of diphenylpicrylhydrazyl radical formation. [c] IC_{50} for inhibition of peroxidation of lipids, estimated as thiobarbituric acid reactive substances. See Materials and Methods for details. nd, not determined.

radical reduction almost completely (>95% of inhibition, data not shown); their I_{50} values were 19.9, 47.9, 30.6, 45.3, 45.2, 38.6, and 41.8 ppm, respectively (**Tables 1** and **2**). The lower I_{50} value for partition E (14.1 ppm) than for any of the fractions from E might be due to a synergistic effect of the components within this extract, similar to that reported for components of *Agrocybe aegerita* fruits (*23*), for which the ethyl acetate fraction was the most active extract for both DPPH radical scavenging and TBARS inhibition.

Of the many biological macromolecules, including carbohydrates, lipids, proteins, and DNA, that can undergo oxidative damage in the presence of ROS, membrane lipids are especially sensitive to oxidation from this physiological process (*24*). For this reason, brain homogenate was used for the investigation of lipid peroxidation as an assessment of oxidative stress. The capacity for plant extracts to prevent lipid peroxidation was assayed using malondialdehyde formation as an index of oxidative breakdown of membrane lipids, following incubation of rat brain cortical homogenate with the oxidant chemical species Fe^{2+}. Ferrous ion both stimulates lipid peroxidation and supports decomposition of lipids peroxides once formed, generating highly reactive intermediates such as hydroxyl radicals, perferryl, and ferryl species (*25*). Partition E was most effective, and partition C was least effective, but none were as effective as quercetin or BHT in inhibiting lipid peroxidation. **Table 1** shows the data that provide IC_{50} values, partition E clearly showing the greatest activity. Thus, partition E reduced lipid peroxidation in a dose-dependent manner and proved to be an excellent antioxidant, reflected by its low IC_{50} value when analyzed by both TBARS and DPPH (**Figure 2**).

When the relative contribution of each fraction to the total antioxidant activity of partition E was evaluated using DPPH and TBARS, all fractions except fraction 1 showed some protective effect, with IC_{50} values between 12.7 and 30.3 ppm (**Table 2**). Fractions 3, 4, 5, and 6 were the most active, with IC_{50} values of 20.1, 23.6, 26.0, and 12.7 ppm, respectively. Fraction 6 was substantially more active than other fractions. It is noteworthy that the value for fraction 6 is very low compared with both values for flavonoids in general, as well as for morin or quercetin (*23*, *26*). We are presently carrying out qualitative and quantitative analyses on this fraction.

It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to the phenolic content (*27*), suggesting a causative relationship between total phenolic content and antioxidant activity (*28*). Halliwell (*1*, *29*) has defined antioxidants as substances that, when present at low concentrations compared with an oxidizable compound (e.g., DNA, protein, lipid, or carbohydrate), delay or prevent oxidative damage due to the presence of ROS. These ROS can undergo a redox reaction with phenolics, such that oxidant activity is inhibited in a concentration-dependent manner. In the presence of low concentrations of phenolics or other antioxidants, the breaking of chain reactions is considered to be the predominant mechanism (*30*), and phenolics have been suggested to be the most active substances from natural sources (*31*). Thus, we measured total phenolic content in each of the extracts, partitions, and fractions (**Figures 3** and **4**). Partition E, which had the greatest DPPH and TBARS activities, had a significantly greater phenolic content than other extracts. The phenolic contents of fractions 1−8 showed a small but significant increase in phenolic content for fraction 5 over fraction 6, which had similar content similar to that of fraction 4; other fractions had significantly lower phenolic contents. These findings correlate well with fraction 5 having the greatest activity against DPPH. Because fraction 6 had hte greatest activity against TBARS formation, it could be that the active component(s) is (are) nonphenolic in nature.

The capacity for a compound to scavenge peroxyl radicals generated by spontaneous decomposition of AAPH was esti-

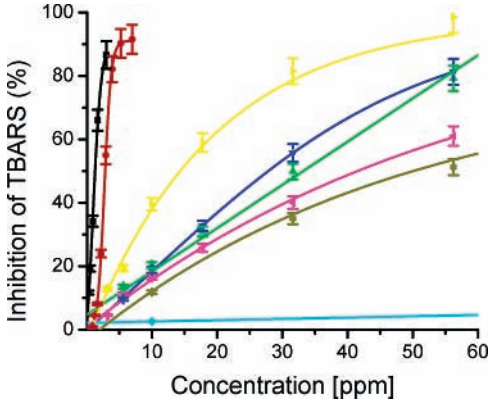


Figure 2. Effects of *P. gentianoides* extracts on the production of TBARS in rat brain homogenate. For explanation of the extracts and partitions, see **Scheme 1**. Extracts: E (yellow), B (dark blue), A (green), D (magenta), and C (olive green). Standard compounds: quercetin (black) and BHT (red).

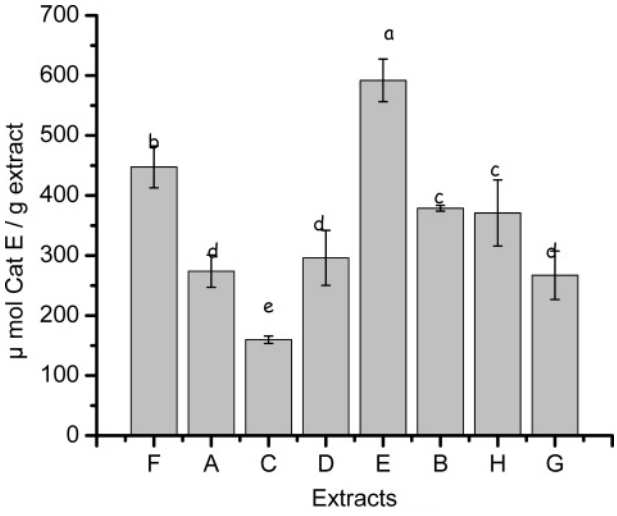


Figure 3. Total phenolic content of *P. gentianoides* and *B. salicifolius* extracts. For explanation of the extracts and partitions, see **Scheme 1**. Values are the mean ± SE of three replicates ($n = 3$); different letters show significant differences at $P < 0.01$, using the Tukey test.

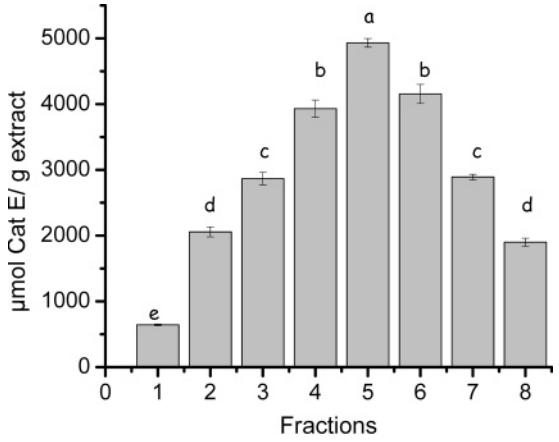


Figure 4. Total phenolic content of fractions from an ethyl acetate partition E of *P. gentianoides*. For explanation of the derivation of fractions, see **Scheme 1**. Each value is the mean ± SE of three replicates ($n = 3$); different letters show significant differences at $P < 0.05$, using the Tukey test.

Table 3. Antioxidant Capacity of *P. gentianoides* and *B. salicifolius* Extracts and Partitions

species	sample[c]	ORAC[a] μg/mL	ORAC[a] μmol of TE/g	FRAP[b] μg/mL	FRAP[b] μmol of Cat E/g of extract
P. gentianoides	F	2.5	1552.4 ± 90.3a	100	161.3 ± 4.1b
	A	2.5	1813.5 ± 148.5a	100	250.0 ± 15.5a
	C	2.5	156.6 ± 25.7c	100	57.1 ± 4.9c
	D	2.5	1808.4 ± 17.0a	200	125.3 ± 5.3b
	E	1.0	3524.8 ± 429.1b	50	377.9 ± 16.7d
	B	2.5	1874.2 ± 43.5a	100	236.3 ± 12.4a
B. salicifolius	H	2.5	1563.9 ± 170.9d	200	78.8 ± 2.7c
	G	2.5	1797.7 ± 168.4a	200	83.1 ± 2.3c

[a] Mean ± SD, $n = 3$. Different letters show significant differences at $P < 0.05$, using Duncan's multiple-range test. [b] Mean ± SD, $n = 3$. Values with the same letter are not significantly different ($P < 0.05$). [c] Extracts F (flower in methanol), A (leaves in methanol), C (leaves in hexane), D (leaves in dichloromethane), E (leaves in ethyl acetate), and B (leaves in water) were from *P. gentianoides*. Extracts H (flowers in methanol) and G (leaves in methanol) were from *B. salicifolius*. For details see **Scheme 1**.

mated in terms of Trolox equivalents, using the ORAC assay (*10*). A wide variety of different phytochemicals from edible plants, purified or as an extract or fraction, have been found to be active in this assay, including alkaloids, coumarins, flavonoids, phenylpropanoids, terpenoids, and phenolic acids (*14*, *32*, *33*). Among the plant extracts assayed here, the values were found to be in the range of 1500−3500 μmol of TE/g of extract for ORAC and from 50 to 400 μmol of Cat E/g of extract for the FRAP assay, respectively (**Tables 3** and **4**). The ORAC and FRAP values for *P. gentianoides* extracts are given in **Table 3**. As with our earlier measurements, partition E had the highest activity in both trials, with values of 3524.8 μmol of TE/g of extract and 377.9 μmol of Cat E/g of extract for ORAC and FRAP assays, respectively. Most of the other extracts all showed values of intermediate potency, ∼1800.0 μmol of TE/g of extract in the ORAC assay, without significantly differing ($P < 0.05$) (**Table 3**). Extract H was somewhat lower, and partition C was essentially without activity. Among the fractions, fraction 5 was significantly more than twice as active as any other fraction (**Table 4**).

The FRAP assay showed greater variability (**Tables 3** and **4**). Several extracts had very low values, and only extracts A and B and partition E showed substantial activity. Again, E was significantly more active than any other sample (**Table 3**). Fractions 4−6, those with substantial phenolic content, were the fractions showing activity in the FRAP assay (**Table 4**). One possible explanation for the many low values obtained is that for these samples, the reaction of the ferric−TPTZ complex was only partially completed within the 10 min reaction period. In agreement with the ORAC assay, it was partitions A, B, and E that showed the greatest values, 250.0, 236.3, and 377.9 μmol of Cat E/g of extract, respectively. In comparison, most fractions showed high FRAP values, with fractions 4, 5, and 6 being the most active values of with 1066.1, 1235.0, and 1086.0 μmol of Cat E/g of extract, respectively (**Table 4**). Those data correlate well with the ORAC values; partition E and fractions 4, 5, and 6 showed the greatest activities of 3524.8, 6165.7, 15501.8, and 5627.7 μmol of TE/g of extract (**Table 4**).

Table 4. Antioxidant Capacity of Fractions from Partition E from *P. gentianoides*, Measured with the ORAC Assay and the FRAP Assay

group[a]	sample (μg/mL)	ORAC[b] (μmol of TE/g of fraction)	sample (μg/mL)	FRAP[c] (μmol of Cat E/ g of fraction)
1	1.0	563.8 ± 40.3a	200	48.7 ± 1.8a
2	1.0	4116.9 ± 183.6b	50	271.4 ± 10.9b
3	1.0	5556.5 ± 379.7c	50	476.2 ± 14.3c
4	1.0	6165.7 ± 636.2c	25	1066.1 ± 57.3d
5	0.5	15501.8 ± 591.8d	25	1235.0 ± 38.7d
6	1.0	5627.7 ± 193.7c	25	1086.0 ± 23.5d
7	1.0	2592.0 ± 262e	50	453.0 ± 16.9c
8	1.0	3069.8 ± 164f	50	415.5 ± 28.1c

[a] Fractions 1–8 from partition E (leaves in ethyl acetate). [b] Mean ± SD, $n = 3$. Different letters show significant differences at $P < 0.05$, using Duncan's multiple-range test. [c] Mean ± SD, $n = 3$. Values with the same letter are not significantly different ($P < 0.05$).

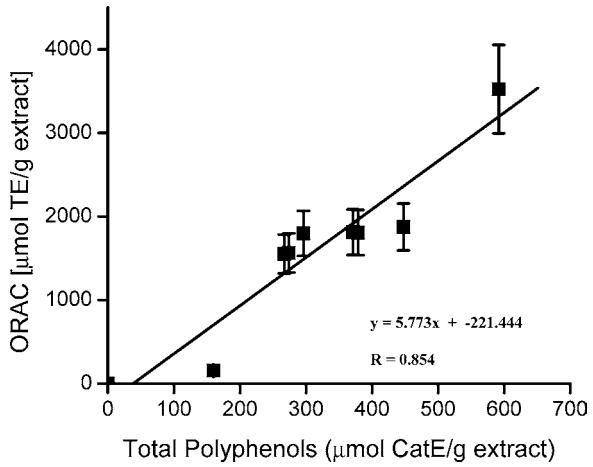


Figure 5. Relationship between total phenolics and ORAC activity for extracts and partitions of *P. gentianoides* [means of three experiments ($n = 3$), $P < 0.01$].

Antioxidant activities bore a direct relationship with the phenolic content of the extracts and fractions. As with DPPH and TBARS activities, extract E was the most active in both the ORAC and FRAP assays. Among the fractions, fraction 5 was the most active in both assays. These facts can be correlated very well between ORAC and total polyphenolic composition of all extracts and partitions and between FRAP and total phenolic composition of fractions, which are shown in **Figures 5** and **6**, respectively. The phenolic characterization suggests that the different phytochemical antioxidant components in the active fractions, still to be determined, may be involved in the

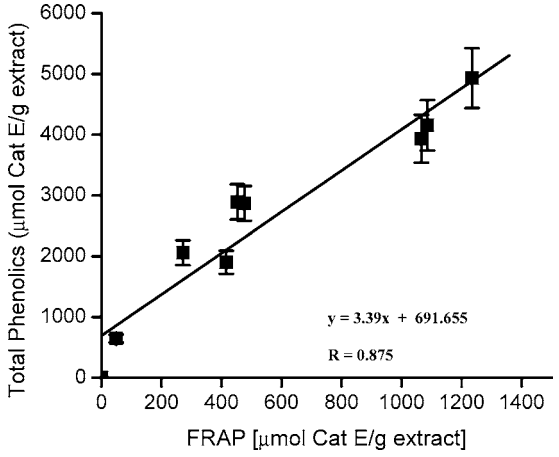


Figure 6. Relationship between total phenolics and FRAP values for fractions of an ethyl acetate extract of *P. gentianoides* [means of three experiments ($n = 3$), $P < 0.01$].

antioxidant mechanism of action, and the ORAC method gives us a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radicals of our samples. Thus, the highest ORAC numbers of our extracts and fractions show an excellent antioxidant potential (**Table 4**), for instance, the fractions 4−6. In addition, the ORAC numbers of fractions showed a very high correlation with polyphenols content ($R >$ 0.9) (data not shown); the same level of correlation was observed between the FRAP numbers and phenolic composition of the extracts and fractions. In the cases of extracts A, B, and E, there is a similar level of correlation ($R > 0.8$) between FRAP numbers and polyphenolic content (data not shown).

Many cellular components are sensitive to oxidative damage caused by the presence of nitrogen or oxygen reactive species, including a myriad of different free radicals. Rat brain homogenates are rich in lipids such as polyunsaturated fatty acids that can undergo peroxidation. Our findings show that the ethyl acetate partition E of *P. gentianoides* and several fractions of that extract contain antioxidants that can inhibit lipid peroxidation and that they have a high phenolic content. The relationship of total phenolics with ORAC and FRAP values in all extracts and fractions was similar to those found in other methanol and ethyl acetate plants extracts, and the values are similar to those for different known fruits and vegetables such as prunes, raisins, blueberries, spinach, and broccoli (*5*, *9*, *23*, *33*−*36*, *41*−*43*).

The ethyl acetate partition E of *P. gentianoides* and some of its fractions exhibited substantial potency in scavenging DPPH radical and inhibiting lipid peroxidation. Four of the eight fractions isolated from E, the fractions 3−6, showed potency in scavenging DPPH radicals, as well as a strong inhibitory effect against lipid peroxidation, particularly fraction 6. The antioxidant activities, total phenolic content, and ORAC and FRAP assays all correlated, suggesting but not proving a causative relationship. That it was the ethyl acetate partition E that showed this activity suggests that the phenolic compounds present are probably low or medium molecular weight, with relatively high polarity. Phytochemical analyses of these extracts, partitions, and fractions are in progress and are expected to identify chemical structures of bioactive components that may have a future role in human health maintenance.

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