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Identification and Quantification of Low Molecular Weight Phenolic Antioxidants in Seeds of Evening Primrose (*Oenothera biennis* L.)

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Crude extracts of evening primrose meal were prepared in 56% (v/v) acetone and separated into six fractions (I−VI) using a Sephadex LH-20 column. Qualitative tests for phenolic and vanillin positive compounds produced positive results for all fractions. Silica gel thin-layer chromatography of fractions III and V allowed the location and isolation of two spots containing moderate to strong antioxidative compounds. High-performance liquid chromatography of the spot isolated from fraction III showed the resolution of two structurally related compounds, whereas that of the spot from fraction V showed the presence of one compound. Nuclear magnetic resonance spectroscopy and electron impact mass spectrometry produced sufficient evidence to identify the isolated compounds as (+)-catechin, (−)-epicatechin, and gallic acid. These compounds accounted for about 10.5 and 1.7% of the dry mass of the crude extracts and meal, respectively.

KEYWORDS: Phenolic antioxidants; evening primrose; chromatography; NMR spectroscopy; electron impact mass spectrometry; catechin; epicatechin; gallic acid

INTRODUCTION

In the past few years, there has been a growing interest in the use of natural additives in preference to synthetic substances for the stabilization of lipid-containing foods (*1*). Because some synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have been implicated in accute toxicity and chronic diseases in experimental animals (*2*), various plant extracts have gained attention as sources of safe antioxidants (*3*). The natural antioxidants in herbs (*4*), spices (*5*), and teas (*6*) have been extensively studied. Various types of oilseeds and their byproducts have also been investigated in the search for safe natural antioxidants. The existing literature on the antioxidants in oilseed meals is voluminous. For instance, the phenolic antioxidants in meals of sesame (*7, 8*), soybean (*9*−*14*), canola (*15*), peanut (*16*−*18*), cottonseeds (*19, 20*), mustard (*21*), and rapeseed (*22*) have been characterized.

The seed oil of evening primrose has attracted interest due to its high content of γ-linolenic acid (~10%, w/w). Oils containing γ-linolenic acid have been used as alternative medications in individuals with heart diseases, eczema, and severe menstrual pain (*23, 24*). The occurrence of γ-linolenic acid in the 1- and 3-positions of the triacylglycerol molecules of evening primrose oil makes them easily accessible to hydrolysis by pancreatic lipase in the small intestines (*25*). Therefore, evening primrose oil is considered to be nutritionally more important than the other seed or vegetable oils containing γ-linolenic acid. As the market for evening primrose oil grows rapidly, a large volume of seeds is being processed to meet the demand and, thus, leading to the production of a large amount of byproducts. The major byproduct of any oilseed processing practice is the spent seeds or meal and, like other oilseed meals, evening primrose meal may also be exploited as a source for natural antioxidants. An extensive investigation on the antioxidant, reactive-oxygen species, and free radical-scavenging properties of crude extracts of evening primrose meal (*26*) was reported and implicated the involvement of phenolic compounds. Lu and Foo (*27*) have also reported antioxidant activity of evening primrose extracts, and the effects were attributed to their phenolic constituents. However, their identification of active compounds was tentative, and the systematic elucidation of chemical structure of active compounds remained to be achieved. Therefore, the objectives of this study were to isolate the low molecular weight antioxidants in evening primrose meal and to elucidate their chemical structures.

MATERIALS AND METHODS

Materials. Seeds of evening primrose (*Oenothera biennis* L.) were obtained from Scotia Pharmaceuticals Ltd., Kentville, NS, Canada. All reagents and authentic standards were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents used in this study were of HPLC grade and were obtained from Fisher Scientific Ltd. (Nepean, ON, Canada). Deuterated acetone and methanol were procured from Cambridge Isotope Laboratories (Andover, MA).

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Preparation of Evening Primrose Meal. Seeds were ground in an electric grinder (Black & Decker Canada Inc., Brockville, ON) for 15 min and then defatted by blending them 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.

Preparation of Evening Primrose Crude Extracts. Crude extracts of evening primrose meal were prepared using 56% (v/v) acetone. The detailed extraction protocol was explained previously (*26*).

Qualitative Detection of Phenolic Compounds. Folin−Denis reagent (0.5 mL) was added to a 50 mL centrifuge tube containing 0.5 mg of crude extracts in 0.5 mL of methanol. The contents were mixed, and 1 mL of a saturated sodium carbonate solution was added into the tube. The volume was then adjusted to 10 mL by the addition of 8 mL of deionized water, and the contents were mixed vigorously. The system was then allowed to stand at ambient temperature for 25 min until the characteristic blue color developed (*28*).

Qualitative Detection of Vanillin Positive Compounds. To 1 mL of methanolic solution containing 1 mg of crude extracts was added 5 mL of freshly prepared 0.5% (w/v) vanillin solution in 4% (w/v) hydrochloric acid, which was mixed vigorously and allowed to stand for 20 min at 30 °C. A positive test was indicated by the characteristic pink color of the solutions (*29*).

Sephadex LH-20 Column Chromatography. 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) packed 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 an LKB Bromma 2112 Redirac fraction collector (Pharmacia, Uppsala, Sweden) and their absorbances read 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 presence or absence of phenolic and vanillin positive compounds in evening primrose fractions was determined as explained previously.

Silica Gel Thin-Layer Chromatography (TLC). Column chromatographic fractions (I−VI) were loaded onto analytical TLC plates (silica gel, 60 Å mean pore diameter, 2−25 mm mean particle size, 250 mm thickness, Sigma Chemical Co., St. Louis, MO). Chromatograms were developed in a glass chamber (22 cm × 22 cm × 10 cm, Fisher Scientific Ltd.) using chloroform/methanol/water (65:35:10, v/v/v) as the mobile phase (*30*). After drying, bands were located by viewing under short (254 nm) and long (365 nm) UV radiation (Spectraline, model ENF-240C, Spectronics Co., Westburg, NY). The following sprays were also used to locate phenolic compounds (spray A) and to examine their antioxidant properties (spray B):

Spray A (Ferric Chloride/Potassium Ferricyanide [$FeCl_3$/$K_2Fe(CN)_6$]). Equal volumes of 1% (w/v) aqueous solutions of each salt were freshly mixed (producing an orange-brown reagent). Phonolics produce blue color with this reagent (*31*).

Spray B (β-Carotene/Linoleate Spray). The relative antioxidant activity of band components on the developed TLC plates was determined using the β-carotene/linoleate spray method (*16*). β-Carotene (9 mg) was dissolved in 30 mL of chloroform. Two drops of linoleic acid and 60 mL of ethanol were added to the β-carotene/chloroform solution. The plates were sprayed with this solution followed by their exposure to fluorescent light for 3 h or until the background orange color disappeared. Bands with persisting orange color were considered to possess antioxidant activity. The color intensity, as judged by the naked eye, was related to their antioxidant strength.

The fractions containing compounds with high antioxidant activity (as determined by β-carotene/linoleate spray) were scraped off and extracted into spectral grade methanol. The slurry was centrifuged (3 min at 5000*g*), and the supernatant was evaporated to dryness under a stream of nitrogen. The dried residues of active components were used for further analysis.

Analytical and Preparative High-Performance Liquid Chromatography (HPLC). A Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-6A pump, an SPD-6A V UV−vis spectrophotometric detector, an SCL-6B system controller, and a CR 501 Chromatopac was used for analytical and preparative HPLC of isolated compounds. Conditions for preparative HPLC were as follows: Hilber prepacked column RT (10 × 250 mm) with Lichrosorb RP-18 (7 μm, Merck, Darmstadt, Germany); water/acetonitrile/methanol/acetic acid (79.5:18:2:0.5, v/v/v/v) as the mobile phase; flow rate of 3 mL/min; injection volume of 500 μL. Pure compounds so obtained were also examined by HPLC separation at ambient temperatures of 25−28 °C on an analytical column. An analytical CWSL column (4.5 × 250 mm) with Spherisorb-ODS-2 (10 μm, Chromatography Sciences Co. Inc., Montreal, PQ, Canada) was used for this purpose. Flow rate was 0.8 mL/min, and the injection volume was 20 μL. For both preparative and analytical HPLC, the detector was preset at 280 nm (*32*).

Ultraviolet (UV) Spectroscopy of Purified Compounds. UV absorption spectra (200−400 nm) of purified compounds (in methanol) were recorded using a Hewlett-Packard diode array spectrophotometer (model 8452A, Hewlett-Packard Co., Mississauga, ON, Canada).

Electron Impact Mass Spectrometry (EI-MS) of Purified Compounds. All mass spectra of purified compounds (in methanol) were recorded using an electron ionization (EI) mode at 70 eV in a 7070 HS Micromass double-focusing mass spectrometer (V.G. Micromass Ltd., Manchester, U.K.). The source, probe, and scanning temperatures were 200, 100−300, and 20−25 °C, respectively.

Proton (^{1}H) and Carbon (^{13}C) Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded using a General Electric 300-NB spectrometer (General Electric, Palo Alto, CA). ^{1}H (at 300 MHz), (^{1}H) correlation spectroscopy (COSY, at 300 MHz), and ^{13}C (at 75.5 MHz) NMR data were collected at room temperature in deuterated methanol (CD_3OD) or deuterated acetone (CD_3COCD_3). Chemical shifts (δ, ppm) were reported relative to tetramethylsilane (TMS) as an internal standard. Data were analyzed using NUTS software (NMR Data Processing Program, Acron NMR Inc., Fremont, CA).

Quantification of Identified Compounds. Once the identification of isolated compounds was achieved, a known mass of each of the fractions was chromatographed on preparative TLC plates. The spots representing the identified compounds were located, scraped off into centrifuge tubes, and then suspended in methanol. The systems were centrifuged at 5000*g* for 3 min, and the compounds were recovered after drying of the supernatants. The yield of each compound was recorded as a weight percent of the crude extracts. Equations used in the calculations are given in the footnotes of **Table 2**.

RESULTS AND DISCUSSION

Sephadex LH-20 Column Chromatography of Crude Extracts of Evening Primrose Meal. Sephadex LH-20 column chromatography has been used by many researchers to fractionate various plant extracts (*30*, *32*−*34*). Sephadex LH-20 is probably one of the best stationary phases available for the separation of phenolics because of their faster, yet satisfactory, separation on it (*15*). As depicted in **Figure 1**, evening primrose crude extracts were separated into six major fractions (I−VI), all of which contained phenolic compounds as they produced the characteristic blue color upon reaction with the Folin−Denis reagent. The presence of catechin and related compounds in evening primrose fractions III−VI was confirmed by a positive vanillin test.

TLC of Evening Primrose Fractions and HPLC of TLC Spots. TLC of evening primrose fractions showed the presence of various phenolic compounds (**Table 1**). All fractions contained phenolics, which did not move from the origin (R_f = 0) and showed strong antioxidant activities because the corresponding spots retained the orange color of the β-carotene even after 3 h of exposure to fluorescent light. These compounds are expected to be high molecular weight oligomers or tannins, and their identification was deferred. The spot with R_f of 0.68 fraction I exerted moderate antioxidant activity, whereas the other two spots contained phenolics with relatively weak antioxidant activities. The spot with R_f 0.88 of fraction II did

Table 1. R_f Values and Antioxidant Activities of Various Evening Primrose Phenolics and Authentic Standards Resolved on Thin-Layer Chromatographic Plates

	spray A	spray B		
fraction	blue[a]	orange[b]	yellow[c]	pale yellow[d]
I	0.00, 0.68, 0.90, 0.97	0.00	0.68	0.90, 0.97
II	0.00, 0.77, 0.88, 0.95	0.00	0.95	0.77
III	0.00, 0.22, 0.53, 0.92	0.00, 0.92		0.53
IV	0.00, 0.22, 0.32	0.00, 0.32	0.22	
V	0.00, 0.13, 0.63	0.00, 0.63	0.13	
VI	0.00, 0.03, 0.06, 0.13, 0.22, 0.32, 0.63	0.00, 0.03, 0.06	0.13, 0.22, 0.63	0.32
gallic acid[e]	0.63	0.63		
(+)-catechin[e]	0.92	0.92		
(−)-epicatechin[e]	0.92	0.92		

[a] Color produced when spray A [1% $FeCl_3/K_2Fe(CN)_6$] was sprayed. [b,c,d] Intensity of color of β-carotene after 3 h from spraying of spray B (β-carotene/linoleate emulsion). [e] Concentration of authentic standard solutions was 1 mg/mL.

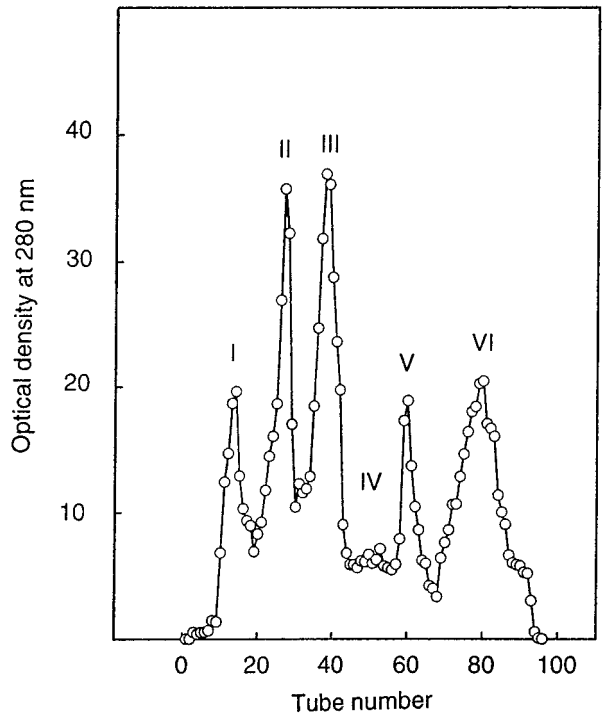

Figure 1. Column chromatographic fraction profile of crude extracts of evening primrose meal.

not retain any of the β-carotene. The other two spots of fraction II had moderate to weak antioxidant activities. The spot with R_f 0.22 of fraction III did not show any antioxidant activity. The spot with R_f 0.92 of fraction III showed a very strong antioxidant activity as evidenced by the delayed bleaching of β-carotene. An intense blue color was also noticed for the spot with R_f 0.92 after it had been sprayed with a 1% (w/v) $FeCl_3/K_2Fe(CN)_6$ solution. The other spot (R_f = 0.53) of fraction III exhibited a weak antioxidant activity. Fraction IV contained phenolics with moderate to high antioxidant activities, but these spots did not produce an intense blue color upon spraying with a 1% (w/v) $FeCl_3/K_2Fe(CN)_6$ solution. Fraction V contained a spot with R_f 0.63 and exhibited a strong antioxidant activity. Fraction VI contained the greatest number of spots with strong to weak antioxidant activities, but their quantities were very small as reflected by the low intensity of blue color produced with 1% (w/v) $FeCl_3/K_2Fe(CN)_6$ spray. Therefore, these spots were not considered for further analysis. The R_f value of one of the spots (0.92) of fraction III corresponded with those of authentic (+)-catechin and (−)-epicatechin. The presence of the flavonoid class of phenolics in crude extracts of evening primrose and fractions III−VI was also confirmed by a positive vanillin test (which produced a pink color). The R_f 0.63 of another spot of fraction V corresponded with that of the authentic gallic acid. The spots corresponding to catechin and gallic acid were isolated on a preparative plate and subjected to analytical HPLC. Analysis of the spot with R_f 0.92 of fraction III, on an analytical HPLC column, showed the presence of two closely related compounds, and these two compounds resolved very close to one another. When these two compounds were resolved on a preparative HPLC column, their retention times were only slightly different. These two compounds were labeled EP-1 and EP-2. The spot with R_f 0.63 of fraction V was a pure compound as its analytical HPLC chromatogram showed a single sharp peak. This compound was labeled EP-3. Compounds EP-1, EP-2, and EP-3 were subjected to spectral analysis to elucidate their chemical structures.

Structural Analysis of Active Components. As described previously, the tentative identities of compounds EP-1, EP-2, and EP-3 were established as (+)-catechin, (−)-epicatechin, and gallic acid, respectively. The following sections will focus on the spectral data of these compounds by means of UV, NMR, and EI-MS.

Spectral Data. *(+)-Catechin (compound EP-1,* ***Figure 2****):* UV, λ_{max} 282 nm; EIMS, *m/z* 290 ($M^{\bullet+}$), *m/z* 152 ($C_8H_8O_3^+$), *m/z* 139 ($C_7H_7O_3^+$), *m/z* 123 ($C_6H_3O_3^+$); ^{1}H NMR (CD_3COCD_3) δ_H 8.00 (4H, s, H_b, H_d, H_l, H_m), 6.88 (1H, d, J = 1.9 Hz, H_n), 6.81 (1H, d, J = 8.1 Hz, H_k), 6.76 (1H, d, J = 8.1 Hz, H_j), 6.01 (1H, d, J = 2.2 Hz, H_c), 5.87 (1H, d, J = 2.2 Hz, H_a), 4.56 (1H, d, J = 8.3 Hz, H_i), 4.00 (2H, pseudo t, J = 8.3, 5.0 Hz, H_h, H_g), 2.91 (1H, dd, J = 16, 5.0 Hz, H_f), 2.55 (1H, dd, J = 16, 8.3 Hz, H_e); ^{13}C NMR (CD_3COCD_3) δ_C 202.9 (C-9), 154.0 (C-7), 153.5 (C-5), 142.0 (C-4′), 141.9 (C-3′), 116.4 (C-5′), 112.0 (C-6′), 111.6 (C-2′), 92.4 (C-6), 91.8 (C-8), 79.0 (C-2), 64.7 (C-3).

(−)-Epicatechin (compound EP-2, ***Figure 2****):* UV, λ_{max} 282 nm; EIMS, *m/z* 290 ($M^{\bullet+}$), *m/z* 152 ($C_8H_8O_3^+$), *m/z* 139 ($C_7H_7O_3^+$), *m/z* 123 ($C_6H_3O_3^+$); ^{1}H NMR (CD_3COCD_3) δ_H 6.96 (1H, d, J = 1.9 Hz, H_n), 6.80 (1H, d, J = 8.1 Hz, H_k), 6.76 (1H, dd, J = 8.1 Hz, H_j), 5.92 (1H, d, J = 2.2 Hz, H_c), 5.90 (1H, d, J = 2.2 Hz, H_a), 4.16 (1H, d, J = 2.2 Hz, H_a), 4.56 (1H, d, J = 8.3 Hz, H_i), 4.00 (2H, pseudo t, J = 8.3, 5.0 Hz, H_h, H_g), 2.91 (1H, d, J = 8.3 Hz, H_i), 3.56 (1H, q, J = inconclusive, H_h), 2.75 (1H, dd, J = 16.0, 5.0 Hz, H_f), 2.75 (1H, dd, J = 16.0, 8.3 Hz, H_e); ^{13}C NMR (CD_3COCD_3) δ_C 153.8 (C-9), 153.5 (C-7), 153.2 (C-5), 141.7 (C-4′), 141.6 (C-

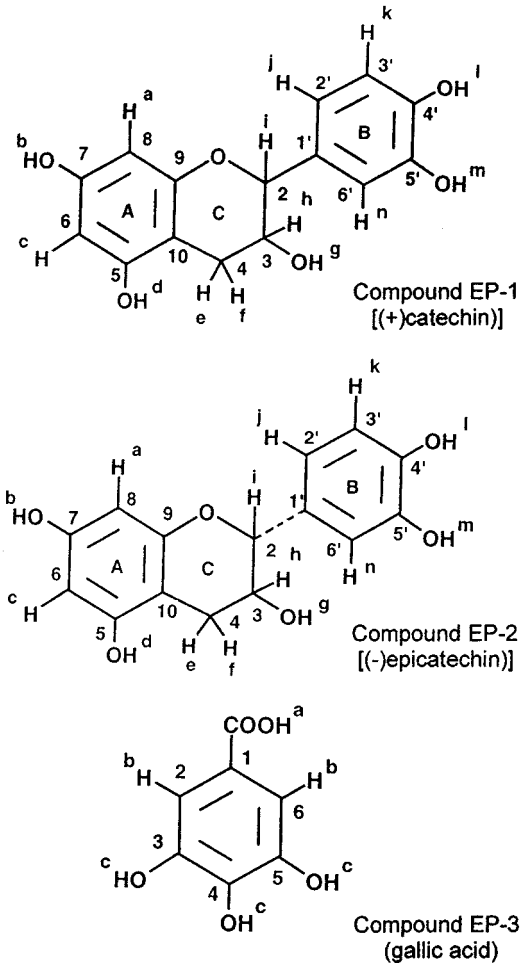

Figure 2. Chemical structures of compounds EP-1, EP-2, and EP-3.

Table 2. Content of Isolated Crude Components in Fractions, Crude Extract, and Meal[a]

compound	% (w/w) in each fraction[b]	% (w/w) in crude extract[c]	% (w/w) in meal[d]
catechins (III)[e]	35.4	10.1	1.62
gallic acid (V)[e]	6.33	0.413	0.066

[a] Results are averages of two runs. Twenty milligrams of each fraction was chromatographed. [b] %, w/w in fraction = (wt of isolated compound in mg/wt of fraction used for TLC in mg) × 100. [c] %, w/w in extract = [(wt of isolated compound in mg × fraction yield in mg)/(wt of fraction used for TLC in mg × wt of extract used for column chromatography)] × 100. [d] %, w/w in meal = [(wt of isolated compound in mg × fraction yield in mg × extract yield in mg)/(wt of fraction used for TLC in mg × wt of extract used for column chromatography × wt of meal used to prepare extract in mg)] × 100. [e] Fraction number.

3′), 128.1 (C-1′), 115.2 (C-5′), 111.1 (C-6′), 111.7 (C-2′), 95.8 (C-10), 92.2 (C-6), 91.2 (C-8), 75.6 (C-2), 63.3 (C-3), 25.1 (C-4).

*Gallic acid (compound EP-3, **Figure 2**):* UV, λ_{max} 272 nm; EIMS, *m/z* 170 ($M^{\bullet+}$), *m/z* 126 ($C_6H_6O_3^+$), *m/z* 108 ($C_6H_4O_2^+$), *m/z* 80 ($C_6H_8^+$), *m/z* 44 (CO_2); ^{1}H NMR (CD_3COCD_3) δ_H 7.04 (2H, s, H_b).

TLC Quantification of Isolated Compounds. Table 2 shows the results for TLC quantification of isolated compounds. (+)-Catechin, (−)-epicatechin, and gallic acid contributed to ∼10.5% (w/w) of the evening primrose crude extracts. Evening primrose meal contained ∼1.7% (w/w) of (+)-catechin, (−)-epicatechin, and gallic acid.

Conclusions. Evening primrose meal contained three major low molecular weight phenolic compounds, namely, (+)-catechin, (−)-epicatechin, and gallic acid. These compounds accounted for ∼10.5 and ∼1.7% of the dry mass of crude extract and meal, respectively. Thus, evening primrose meal may be exploited as a source of bioactive components for use in nutraceutical as well as nonfood applications.

ABBREVIATIONS USED

TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; UV, ultraviolet; EI-MS, electron impact mass spectrometry; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; TMS, tetramethylsilane; δ, chemical shift; ppm, parts per million; $M^{\bullet+}$, molecular radical cation.

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