

E*-Cinnamic Acid Derivatives and Phenolics from Chilean Strawberry Fruits, *Fragaria chiloensis* ssp. *chiloensis

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Three *E*-cinnamic acid glycosides, tryptophan, and cyanidin-3-*O*- β -D-glucopyranoside were isolated from ripe fruits of the Chilean strawberry *Fragaria chiloensis* ssp. *chiloensis*. 1-*O*-*E*-Cinnamoyl- β -D-xylopyranoside, 1-*O*-*E*-cinnamoyl- β -D-rhamnopyranoside, and 1-*O*-*E*-cinnamoyl- α -xylofuranosyl-(1 \rightarrow 6)- β -D-glucopyranose are reported for the first time. The cinnamic acid glycosides and aromatic compound patterns in *F. chiloensis* fruits were determined by high-performance liquid chromatography (HPLC). HPLC analyses of extracts showed that cyanidin-3-*O*- β -D-glucopyranoside and free ellagic acid are present in achenes while the *E*-cinnamoyl derivatives and tryptophan were identified only in the thalamus. The free radical scavenging effect of the fruit extract can be associated with the anthocyanin content.

KEYWORDS: *Fragaria chiloensis*; Chilean strawberry; *E*-cinnamic acid derivatives; free radical scavengers; antioxidants; anthocyanin

INTRODUCTION

Fragaria chiloensis is one of the progenitors of the widely cultivated strawberry (*F. × ananassa*), the most important of the cultivated strawberries. *F. chiloensis* ssp. *chiloensis* is endemic to southern Chile and can also be found in some parts of Peru and Ecuador (1). Recent recognition of the narrow genetic base of cultivated strawberries (2) and the need for biotic and abiotic stress genes has made wild accessions of *F. chiloensis* an important germplasm source for the further development of domesticated crops. In addition, the potential for exploiting *F. chiloensis* as a crop in its own right is another realistic possibility.

Little is known of the chemistry of the wild strawberry progenitors. Sugar esters of hydroxycinnamic acid have been identified in several strawberry cultivars (3). However, little attention was paid to the glycoconjugates of the unsubstituted precursor, cinnamic acid. Glycosylated cinnamic acid derivatives have been shown to be potential flavor precursors (4) and to have allelochemical capacity (5).

As we were unable to find reports on the identity and antioxidant activity of phenolics from the white fruited form of the Chilean strawberry *F. chiloensis*, the ripe fruit from a semicommercial plantation in south Chile was investigated. The

isolation and characterization of aromatic compounds from a polar extract of *F. chiloensis* fruits were undertaken as well as the antioxidant capacity and distribution of these compounds in thalamus and achenes.

MATERIALS AND METHODS

Reagents. All solvents used were of analytical grade. Chloroform was purchased from Fisher (Fairlawn, NJ), and ethanol and methanol were obtained from J. T. Baker (Phillipsburg, NJ). 1-Butanol and acetic acid were purchased from Caledon Lab. (Georgetown, Canada) and Merck (Darmstadt, Germany), respectively. High-performance liquid chromatography (HPLC) grade acetonitrile and formic acid from Merck were used. The standards of monosaccharides were purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography (TLC) analysis was carried out on aluminum-coated silica gel (Sigma-Aldrich, St. Louis, MO) and Cellulose F254 plates from Merck. Folin–Ciocalteu phenol reagent, aluminum chloride hexahydrate, and sodium carbonate were from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetin, nitro blue tetrazolium (NBT), thiobarbituric acid, xanthine oxidase, hypoxanthine, and neutral red were purchased from Sigma Chemical Co. Culture media, antibiotics, and fetal bovine serum were obtained from Invitrogen Corp. (Carlsbad, CA).

Plant Material. Ripe *F. chiloensis* ssp. *chiloensis*, the white-fruited form, was harvested in a commercial plantation located in Pichihuillico, Contulmo, Province of Arauco, VIII Region, Chile (S 38° 04' 8.6", W 73° 14' 2.96") at 605 m above sea level. The strawberries were sorted to eliminate damaged, poor quality fruit and to obtain a uniform sample in size and color. After that, the samples were immediately frozen at –80 °C until extraction. A voucher herbarium specimen was deposited with the number 2865 in the Herbarium of the Universidad de Talca.

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Total Phenolic and Flavonoid Content. A precisely weighed amount of whole fruit, pulp, or achenes from *F. chiloensis* was homogenized for 5 min and extracted with 1% HCl in methanol (MeOH) (2 mL/0.01 g of achenes and 2 mL/g of pulp). The extracts were mixed and allowed to stand for 1.5 h at room temperature. The extracts were filtered through Whatman filter paper, and the filtrates were taken to a final volume with distilled water. Extractions were carried out in triplicate. The extracts obtained were used to determine total phenolic and flavonoid content as well as HPLC profiles. The total phenolic contents of achenes, thalamus, and whole fruit were determined using the method described by Singleton et al. (6). Briefly, the appropriate extract dilution was oxidized with the Folin–Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 700 nm after 30 min using a HeLIos α V-3.06 UV/vis spectrophotometer. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg gallic acid equivalents (%). The total flavonoid content in the samples was determined by the methodology of Chang et al. (7), based on the principle that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the *ortho*-dihydroxyl groups in the A or B ring of flavonoids. Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as mg quercetin equivalents (%). Data are reported as means \pm SD for at least three replicates.

Total Anthocyanin Content. For the total anthocyanin content, acetone extracts of whole fruit, achenes, and thalamus were prepared. The extracts were filtered through Whatman filter paper, and the filtrates were taken to a final volume (5 mL) with distilled water. The total anthocyanin content of the acetone extract was measured using a modified pH differential method described by Meyers et al. (8). A HeLIos α V-3.06 UV/vis spectrophotometer was used to measure absorbance at 510 and 700 nm in buffers at pH 1.0 and 4.5. Absorbance readings were converted to total mg of cyanidin 3-glucoside equivalents per 100 g fresh weight of strawberry using the molar extinction coefficient of 26900 and an absorbance of $A = [(A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}]$. Data were reported as means \pm SD for three replications.

Extraction and Isolation of Aromatic Compounds. A 780 g amount of fresh fruit was cut into pieces and homogenized in a Waring blender. The homogenized sample was then transferred to an Erlenmeyer flask and extracted with acetone (2 L) for 48 h in the dark, as recommended by Degenhardt et al. (9) and Kong et al. (10). The slurry was filtered, and the filtrate was concentrated under reduced pressure at temperatures lower than 40 °C. A representative sample was lyophilized; the w/w yield in terms of fresh starting material was 5.49%. The aqueous extract was partitioned with an equal volume of ethyl acetate (EtOAc) to afford an EtOAc soluble fraction (0.14% w/w) and an aqueous fraction. A 300 mL amount of the aqueous fraction was diluted with distilled water to 1000 mL, filtered, and passed through an Amberlite XAD-7 column according to the procedure described by Degenhardt et al. (9) with some modifications. The column was rinsed with water and washed with MeOH (2 L). The eluate was concentrated under reduced pressure, and a representative sample was lyophilized. The w/w yield of the eluate in terms of fresh starting material was 0.23%. A 1.8 g amount of the Amberlite-retained extract was permeated through a Sephadex LH-20 column (column length 29 cm, internal diameter 3 cm) using MeOH:H₂O (9:1) as the mobile phase; 45 fractions of 5 mL each were obtained. After TLC comparison on silica gel (EtOAc:AcOH:H₂O, 10:2:3, v/v/v), fractions with similar patterns were pooled into three groups. The pooled fractions 6–10 (126 mg, pool A), 11–16 (92 mg, pool B), and 17–23 (130 mg, pool C) were submitted to high-speed countercurrent chromatography (HSCCC) for the isolation of their components.

The HSCCC instrument (P. C. Inc., Potomac, MD) was equipped with a multilayer coil of 1.68 mm i.d. polytetrafluoroethylene (PTFE) tubing of approximately 80 and 240 mL with a total capacity of 320 mL. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge (*R*), was 10.5 cm, and the β value

was 0.76 ($\beta = r/R$, where *r* is the distance from the coil to the holder shaft). The speed varied between 0 and 1200 rpm. The flow rate was controlled with a DC Analytic Gearmotor (Bodine Electric Company, Chicago, IL). The sample was injected with a P. C. Inc. injection module with a 10 mL sample injection loop. The solvent system used was *n*-BuOH:AcOH:H₂O (4:1:5, v/v/v) as previously reported (11). It was thoroughly equilibrated overnight in a separation funnel at room temperature, and the two phases were separated and degassed, by sonication, shortly before use. The solvent system provided an ideal range of the partition coefficient (*K*) for the applied sample and a desirable settling time (28 s); 33% of the stationary phase was retained in the coil. First, the coiled column was entirely filled with the stationary phase (upper phase, 320 mL). Then, the apparatus was rotated forward at 800 rpm, while the mobile phase (lower phase) was pumped into the column in a head to tail (H \rightarrow T) direction at a flow rate of 2.5 mL/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 10 mL of filtered sample solution (sample, dissolved in 5 mL of upper phase and 5 mL of lower phase) was injected through the injection module. The effluent of the column was continuously monitored by TLC on silica gel, (mobile phase EtOAc:AcOH:H₂O, 10:2:3, v/v/v). The spots were observed under ultraviolet light (254 nm).

From the pool A fraction, HSCCC afforded 50 fractions of 8 mL each. A 38.5 mg amount of compound **1** (retained in the stationary phase) and 5.8 mg of compound **2** (elution volume 160–224 mL) were obtained. HSCCC of the pool fraction C yielded 50 fractions of 8 mL each, which, after TLC comparison, gave 17.6 mg of compound **5** (elution volume, 136–176 mL). HSCCC of the pool B yielded 40 fractions of 8 mL each. A 5 mg amount of compound **3** (retained in the stationary phase) and 19 mg of compound **4** (elution volume 104–144 mL) were obtained. Ellagic acid, compound **6**, was identified from the Amberlite-retained crude extract by TLC and DAD-HPLC comparison with a standard sample (*R_f* and UV spectrum).

Structural Identification of the Compounds. Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker spectrometer, operating at 400 MHz for ¹H and 100 MHz for ¹³C. Methanol (CD₃-OD) and D₂O were used as solvents. The anthocyanin spectra were recorded in CD₃OD/CF₃COOD (19:1, v/v). Chemical shifts are presented in ppm and the coupling constant (*J*) in Hz. The UV/vis spectra were obtained using a HeLIos α V-3.06 spectrophotometer; MeOH was used as solvent. HPLC-DAD was used for the identification of the main compounds in the crude extracts, fractions, and as a purity criterion for the isolated compounds before NMR measurements and assays.

Compounds **2**, **3**, and **5** (2 mg each) were subjected to acid hydrolysis (2 M HCl, 40 min, 100 °C) by refluxing. The aglycones were extracted with EtOAc and subjected to TLC analysis using as mobile system EtOAc:AcOH:H₂O (10:2:3, v/v/v). The spots on the TLC plates were observed under ultraviolet light (254 nm) and revealed with diphenyl boric acid/ethanolamine complex in methanol. The sugars in the aqueous solution were identified by TLC on cellulose with known sugars standards. As a mobile phase, *n*-BuOH–benzene–pyridine–H₂O (5:1:3:3, v/v/v/v) was used. The spots on the TLC plates were revealed with aniline hydrogen phthalate.

Compound 1: *1-O-E-Cinnamoyl- β -D-rhamnopyranoside*. Elemental analysis calcd for C₁₅H₁₈O₆: C, 61.22; H, 6.16. Found: C, 61.04; H, 6.14. [α]_D²⁰ + 37.5 (*c* = 0.4, H₂O). UV (λ_{max} 284 nm, HPLC-DAD). The structure of compound **1** followed from the NMR data, which clearly showed a cinnamoyl unit and a sugar moiety. The sugar was identified as rhamnose by its ¹H and ¹³C NMR spectra and was attached at the C-1 position as shown by the chemical shift of the anomeric H at δ 5.59 (d, *J* = 7.6 Hz), in full agreement with the corresponding ¹³C NMR signals. Acid hydrolysis afforded cinnamic acid and rhamnose. The α -rhamnopyranoside was previously obtained by precursor-directed biosynthesis with *Streptomyces griseoviridis* (12).

Compound 2: *1-O-E-Cinnamoyl- α -xylofuranosyl-(1 \rightarrow 6)- β -D-glucopyranose*. Elemental analysis calcd for C₂₀H₂₆O₁₁: C, 54.30; H, 5.92. Found: C, 54.08; H, 5.90. UV (λ_{max} 284 nm, HPLC-DAD). The ¹H NMR spectrum clearly indicated a cinnamoyl unit and two sugars. One of them had an anomeric proton at δ 5.58 with a coupling constant of 8 Hz, supporting a β -configured sugar while the signal at δ 4.93 agreed

Table 1. ¹H NMR Data of Compounds 1–3 (400 MHz, δ Values), J (Hz) in Parentheses^a

H	1 (MeOH-d ₄ + D ₂ O)	2 (MeOH-d ₄ + D ₂ O)	3 (DMSO-d ₆)
2	6.58 d (16)	6.58 d (16)	6.65 d (16)
3	7.81 d (16)	7.81 d (16)	7.74 d (16)
5, 9	7.63 m	7.64 m	7.74 m
6, 7, 8	7.43 m	7.42 m	7.44 m
sugar	rhamnose	glucose	xylose
1'	5.59 d (7.6)	5.58 d (8)	5.47 d (8)
2'	3.42 m	3.44 dd (8, 8)	} 3.15–3.40 m
3'	3.68 m	} 3.34–4.04	
4'	3.6–3.8 m		
5'	3.77 m		
6'	1.13 d (6.4)		
1''			4.93 s
2''–5''		3.34–4.04	

^a Overlapping signals.

well with an α-configured sugar unit. After acid hydrolysis, xylose and glucose were identified by TLC analysis. The sequence and position of the sugars followed from the 2D NMR experiments. While the anomeric proton at δ 5.58 showed correlation with the carbonyl group of the cinnamic acid at δ 164.8 ppm, the second anomeric signal appeared as a br s at δ 4.93 and present interactions with the C at δ 83.4, 76.6, and 65.7 ppm, which correspond to the C-2 and C-3 of the second sugar and the t at 65.7 of the first sugar unit. The proton signal at δ 5.58 resonated as a d (*J* = 8 Hz) and coupled with a dd at δ 3.44 ppm (*J* = 8, 8 Hz) in agreement with the expected multiplicities for the glucose moiety. The interglycosidic linkage between the terminal xylose and the inner glucose was deduced as (1→6) on the basis of the HMBC correlations. A closely related compound, 1-*O*-*E*-cinnamoyl-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranose, has been previously reported from *Psidium guajava* (13, 14).

Compound 3: 1-*O*-*E*-Cinnamoyl-β-D-xylopyranoside. Elemental analysis calcd for C₁₄H₁₆O₆: C, 60.00; H, 5.75. Found: C, 59.88; H, 5.74. [α]_D²⁰ + 5.88 (*c* = 3.4, H₂O). UV (λ_{max} 284 nm, HPLC-DAD). The ¹H NMR spectrum of compound 3 showed two multiplets at δ 7.44 and 7.74, which integrated for 5 H, indicating a monosubstituted aromatic ring. The *trans*-olefinic protons at δ 7.74 and 6.65 (*J* = 16 Hz) were in agreement with a cinnamic acid derivative. The identity of the sugar followed from the ¹H and mainly the ¹³C NMR spectrum, which matched with the reported data for β-D-xylopyranoside (15). Acid hydrolysis confirmed the sugar identity.

The ¹H NMR data of compounds 1–3 are presented in **Table 1**, while the ¹³C NMR data are summarized in **Table 2**.

Compound 4: Tryptophan. The ¹H and ¹³C NMR data were in full agreement with a standard sample.

Compound 5: Cyanidin-3-*O*-β-D-glucopyranoside. The ¹H and ¹³C NMR data as well as the visible and UV spectra were in agreement with literature values reported by Du et al. (16).

The structures of the isolated compounds 1–3 and 5 are presented in **Figure 1**.

HPLC Analysis of Isolated Compounds. HPLC analysis was performed using HPLC-DAD Merck-Hitachi (LaChrom, Tokio, Japan) equipment consisting of a L-7100 pump, a L-7455 UV diode array detector, and D-7000 chromatointegrator. A 250 mm × 4.6 mm i.d., 5 μm C18-RP column (Phenomenex, Torrance, CA) was used. The compounds were monitored at 256 nm, and the absorbance was measured between 200 and 400 nm. Gradient elution was carried out with water:0.1% formic acid (solvent A) and 20% solvent A in 80% acetonitrile (solvent B) at a constant flow rate of 1 mL/min according to Sánchez-Rabanaeda et al. (17). In the case of anthocyanins, monitoring was at 521 nm and the absorbance was measured between 500 and 560 nm. The solvent system was a linear gradient from 100% A (17.6% formic acid) to 50% A in 15 min, followed by 5 min of 100% B (acetonitrile) at a flow rate of 1 mL/min according to Einbond et al. (18).

DPPH Discoloration Assay. The free radical scavenging effect of the extracts and compounds was assessed by the discoloration of a

Table 2. ¹³C NMR Data of the Compounds 1–3 (100 MHz, δ Values)

C	1 (MeOH-d ₄ + D ₂ O 3:1)	2 (MeOH-d ₄ + D ₂ O 1:1)	3 (DMSO-d ₆)
1	168.0 s	164.8 s	167.0 s
2	118.2 d	116.0 d	117.2 d
3	149.4 d	145.5 d	148.2 d
4	135.0 s	129.6 s	136.3 s
5, 9	130.1 d	127.5 d	130.0 d
6, 8	130.0 d	127.2 d	130.1 d
7	133.9 d	133.3 d	134.1 d
sugar	rhamnose	glucose	xylose
1'	96.0 d	93.0 d	94.4 d
2'	71.1 d	71.8 d	76.2 d
3'	74.0 d	75.6 d	77.1 d
4'	78.9 d	66.9 d	72.3 d
5'	69.2 d	76.3 d	69.1 t
6'	23.8 q	65.7 t	
		xylose	
1''		107.8 d	
2''		83.4 d	
3''		76.6 d	
4''		81.0 d	
5''		54.4 t	

methanolic solution of DPPH as previously reported (19). Crude extracts were assayed at 100, 33, and 11 μg/mL, and pure compounds were assayed at 10, 3.3, and 1.1 μg/mL. The scavenging of free radicals by extracts and compounds was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical.

The percentage of discoloration was calculated as follows:

$$\% \text{ of discoloration} = 1 - \frac{\text{absorbance of compound/extract}}{\text{absorbance of blank}} \times 100$$

The degree of discoloration indicates the free radical scavenging efficiency of the substances. The half-maximal inhibitory concentration (IC₅₀) was calculated by linear regression analysis. Quercetin was used as a free radical scavenger reference compound. Values are reported as means ± SD of three determinations.

Superoxide Anion. The enzyme xanthine oxidase is able to generate superoxide anion by oxidation of reduced products from intracellular ATP metabolism. In this reaction, the xanthine oxidase oxidizes the substrate hypoxanthine generating superoxide anion, which reduces the NBT dye, leading to a chromophore with absorption maxima at 560 nm. Superoxide anion scavengers reduce the generation speed of the chromophore. The activity was measured spectrophotometrically as reported previously (19) using a Genesys-10 UV scanning spectrophotometer. Extracts were evaluated at 50 μg/mL, and compounds were evaluated at 50, 25, and 10 μg/mL. Values are presented as means ± SD of three determinations. The activity of the pure compounds is presented as mean IC₅₀ ± SD, calculated by linear regression analysis. Quercetin was used as a reference compound. The percentage of superoxide anion scavenging effect was calculated as follows:

$$\% \text{ of scavenging activity} = \frac{E - S}{E} \times 100$$

where *E* = *A* - *B* and *S* = *C* - (*B* + *D*); *A* is the optical density of the control; *B* is the optical density of the control blank; *C* is the optical density of the sample; and *D* is the optical density of the sample blank.

Lipoperoxidation in Erythrocytes. Studies on erythrocytes lipid peroxidation were carried out as previously described (19). Extracts and compounds were tested at 50 μg/mL. Human red blood cells obtained from healthy donors were washed three times in cold phosphate-buffered saline (PBS) by centrifugation at 1100g for 5 min. After the last washing, cells were resuspended in PBS and their density was adjusted to 1 mM hemoglobin in each reaction tube. The final cell suspensions were incubated with different concentrations of the test compounds dissolved in DMSO and PBS during 5 min at 37 °C. The final concentration of DMSO in the samples and controls was 1%. After incubation, cells were exposed to *tert*-butylhydroperoxide (1 mM)

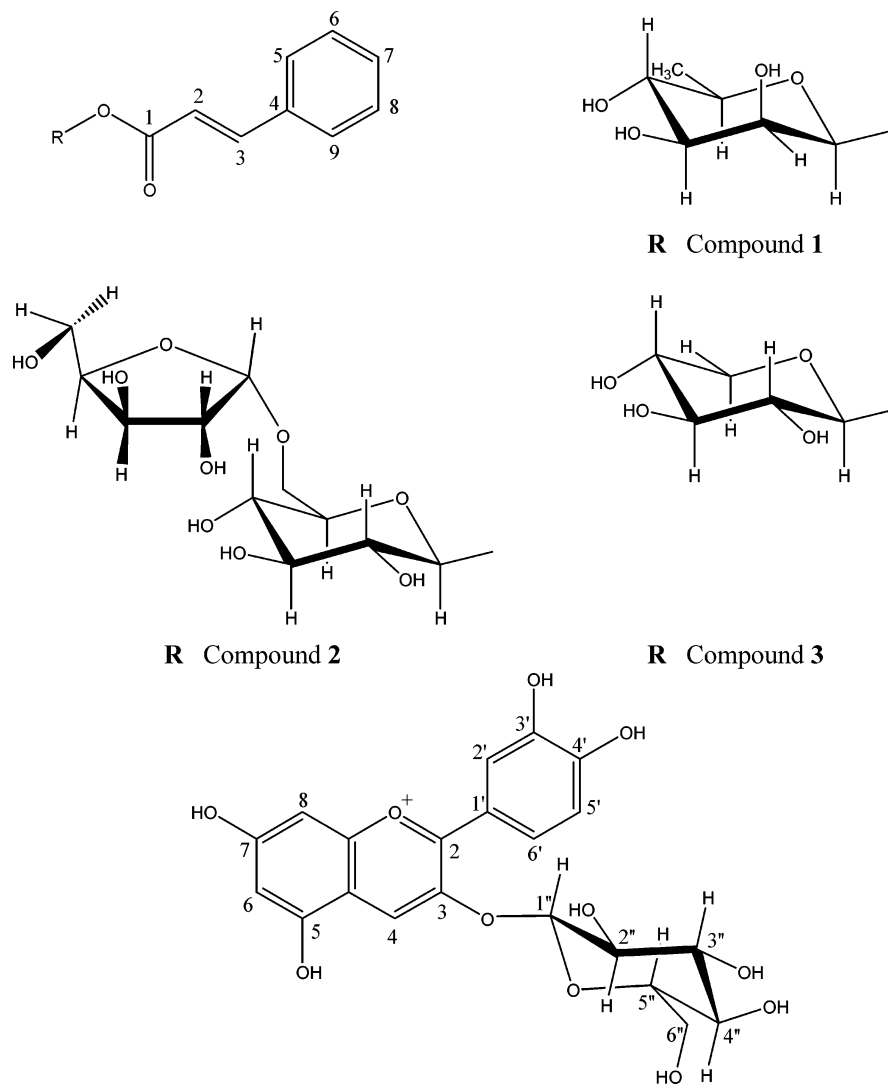


Figure 1. Chemical structures of the compounds 1–3 and 5.

during 15 min at 37 °C under vigorous shaking. After treatment, lipid peroxidation was determined indirectly by the thiobarbituric acid reactive substance (TBARs) formation. Results are expressed as percentages of inhibition as compared to controls. The percentage of inhibition of the formation of TBARs (% inhibition of lipid peroxidation) was calculated as follows:

$$\% \text{ inhibition of lipid peroxidation} = \frac{(A_1 - A_t)}{(A_1 - A_2)} \times 100$$

where A_1 , A_2 , and A_t are the absorbance values at 535 nm for the unprotected samples, the blanks, and the test samples, respectively.

Cytotoxicity. The cytotoxic effect of the compounds, expressed as cell viability, was assessed on a permanent fibroblast cell line derived from human lung (MRC-5) (ATCC no. CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM), with Earle's salts, 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin in a humidified incubator with 5% CO₂ in air at 37 °C. Cells were plated at a density of 2.5×10^3 per well in 96 well plates. Confluent cultures of MRC-5 cells were treated with medium containing the compounds at concentrations ranging from 80 up to 1000 μ M. The substance was first dissolved in DMSO and then in MEM. The final concentration of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to test medium with or without the compound (control). Each drug concentration was tested in quadruplicate and repeated three times in separate experiments. At the end of incubation, the neutral red uptake

assay was carried out as described by Rodríguez and Haun (20). To calculate the IC₅₀ values, the results were transformed to percentage of controls and the IC₅₀ values were graphically obtained from the dose–response curves.

Statistical Analysis. To determine whether there was any difference between activity or phenolics content of samples, one-way analysis of variance test was applied. Values of $P < 0.05$ were considered as significantly different. The differences among means were determined using Tukey's multiple comparison test. The Statistical Package S-Plus 2000 for Windows was used to analyze the data.

RESULTS AND DISCUSSION

The commercial strawberry (*Fragaria* spp.) has been shown to have a narrow genetic base (21) leading to “detrimental inbreeding effects”, limited tolerance of environmental fluctuations (2), and reduced potential for crop improvement. Disease and pests are often the limiting factors with regard to production and fruit quality. The use of germplasm from wild relatives may help reduce the use of costly chemicals for disease and pest control and also increase production efficiency (22). Therefore, the investigation of wild strawberry species can afford valuable information for new breeding programs.

Six aromatic compounds, including ellagic acid, *E*-cinnamic acid glycosides, an amino acid, and an anthocyanin, were isolated from the Chilean strawberry (*F. chiloensis* ssp. *chiloensis*). The *E*-cinnamic acid derivatives (compounds 1–3) are

Table 3. Percentage of Activity Relative to the Corresponding Control Induced by *F. chiloensis* Extracts and Compounds on Free Radical and Superoxide Anion Scavenging and Inhibition of Lipid Peroxidation^a

samples	discoloration of DPPH (%)	superoxide anion scavenging (%)	inhibition of lipid peroxidation (%)
extracts	10 μ g/mL	50 μ g/mL	50 μ g/mL
crude	12.0 \pm 0.7	44.4 \pm 0.2	34.3 \pm 4.0
EtOAc	63.2 \pm 1.4	52.1 \pm 0.3	16.5 \pm 2.1
retained in Amberlite	88.3 \pm 0.7	60.0 \pm 0.1	81.7 \pm 0.1
compounds	IC ₅₀ μ g/mL		
1 : 1- <i>O</i> - <i>E</i> -cinnamoyl- β -D-rhamnopyranoside	114.7 \pm 1.2	13.0 \pm 0.4	21.4 \pm 5.9
2 : 1- <i>O</i> - <i>E</i> -cinnamoyl- α -xylofuranosyl-(1 \rightarrow 6)- β -D-glucopyranose	134.8 \pm 1.1	34.2 \pm 0.2	11.1 \pm 2.7
3 : 1- <i>O</i> - <i>E</i> -cinnamoyl- β -D-xylopyranoside	78.9 \pm 0.4	24.4 \pm 0.5	inactive
4 : tryptophan	73.3 \pm 0.4	30.2 \pm 0.2	39.6 \pm 5.0
5 : cyanidin-3- <i>O</i> - β -D-glucopyranoside	7.3 \pm 0.1	51.2 \pm 1.5	ND
6 : ellagic acid	1.2 \pm 0.2	ND	ND
quercetin	2.6 \pm 0.2	85.9 \pm 2.5	80.7 \pm 0.9

^a Results are presented as means \pm SD. All values were significantly different ($P < 0.05$). ND, not determined due to insolubility of the sample.

reported for the first time. *E*-Cinnamoyl- α -D-rhamnopyranoside was obtained by biosynthesis with *S. griseoviridis* (12), and the corresponding glucopyranoside has been reported from developing strawberry fruit (*Fragaria ananassa*) (14). 1-*O*-*E*-Cinnamoyl- β -D-glucose was also isolated from *P. guajava*, *Salix sacchalinensis*, *Spiraea thunbergii*, and *Vaccinium vitis-idaea*. The corresponding diglucoside, 1-*O*-*E*-cinnamoyl- β -D-gentiobioside, was reported from *Physalis peruviana* (13, 23). The closely related arabinosylglucoside has been previously reported from *P. guajava* (13). According to Groyne et al. (4), 1-*O*-*E*-cinnamoyl- β -D-glucopyranose would be a possible precursor of volatile cinnamates in strawberry flavor. Methyl and ethyl cinnamate were identified as volatile compounds in *F. ananassa* fruits (24).

The cinnamoyl glucosides 1-*O*-*Z*-cinnamoyl- β -D-glucopyranose and 6-*O*-(4'-hydroxy-2'-methylene-butyryl)-1-*O*-*Z*-cinnamoyl- β -D-glucopyranose were described as major plant growth inhibitory constituents from *S. thunbergii* Sieb. (5). The related compounds 6-*O*-(*E*-cinnamoyl)-1-*O*-(4'-hydroxy-3'-methyl-furan-2'-one)- β -D-glucopyranose, 6-*O*-(4'-hydroxy-2'-methylene-butyryl)-1-*O*-*E*-cinnamoyl- β -D-glucopyranose, and 1-*O*-*E*-cinnamoyl- β -D-glucopyranose presented lower phytotoxicity. The growth inhibitory activity, however, was detected mainly in the *Z*-isomers. According to Hiradate et al. (5), it would be possible that *S. thunbergii* accumulates *E*-cinnamoyl glycosides in detoxified forms and secretes *Z*-cinnamoyl glycosides as the toxic form of effective allelochemicals. Possible pathways for *Z*-cinnamic acid synthesis include sunlight-mediated conversion from *E*-cinnamic acid, spontaneous conversion from *E*-cinnamic acid in the presence of electron transfer sensitizer, isomerase-mediated conversion from *E*-cinnamic acid, and direct enzymatic biosynthesis from L-phenylalanine.

According to Gil et al. (25), the main anthocyanin found in strawberries is pelargonidin 3-glucoside, with cyanidin 3-glucoside and pelargonidin 3-rutinoside present as minor components. In our study, cyanidin-3-*O*-glucopyranoside was the main anthocyanin in *F. chiloensis* whole fruits. Ellagic acid is one of the main phenolics in *Fragaria* berries (26).

The crude acetone extract, the EtOAc fraction, and the Amberlite-retained material were assessed for free radical scavenging activity by the DPPH discoloration assay and the superoxide anion test as well as by the inhibition of lipid peroxidation in erythrocytes. The most active fraction in all assays was the Amberlite-retained mixture (Table 3). From the Amberlite-retained fraction, the highly active free radical scavenger cyanidin-3-*O*- β -D-glucopyranoside was isolated, hav-

ing an IC₅₀ value of 7.3 μ g/mL in the DPPH discoloration assay and 51.2% superoxide anion scavenging effect at 50 μ g/mL. As expected by their structures, compounds 1–4 were weakly active as free radical scavengers, indicating that the observed effect found in the polar extract was most probably related to the anthocyanin and ellagic acid content. All of the isolated compounds proved to be devoid of toxicity toward human lung fibroblasts even at concentrations as high as 1 mM.

Wang et al. (27) reported cyanidin as the most widespread anthocyanidin and 3-glucoside as the most active antioxidant anthocyanin. Cyanidin 3-*O*- β -glucoside showed the highest oxygen radical absorbance capacity (ORAC) among the 14 anthocyanins tested, and its effect was 3.5 times stronger than that of the vitamin E analogue Trolox. Similar results were obtained by Kähkönen et al. (28). Tsuda et al. (29) found that cyanidin 3-*O*- β -glucoside can lower the serum TBARs concentration and increase the oxidation resistance of the serum to lipid peroxidation in rats. According to Fukumoto and Mazza (30), cyanidin 3-*O*- β -glucoside has been found to be twice as effective as commercially available antioxidants, such as butylated hydroxyanisole and α -tocopherol. Amorini et al. (31) confirmed the remarkable antioxidant capacity of cyanidin 3-*O*- β -glucoside in the model of Cu²⁺-mediated human low density lipoprotein oxidation, which was higher than both resveratrol and ascorbic acid. Ellagic acid has been shown to function as an antioxidant (32), and there is considerable interest in the potential anticancer effects of this compound (33).

The pattern of compounds bearing an aromatic moiety in *F. chiloensis* fruits was determined by HPLC. Under our experimental conditions, the *R*_t (min) of the isolated compounds was as follows: compound 1, 55.0 min; compound 2, 48.5 min; compound 3, 49.3 min; compound 4, 28.4 min; compound 5, 10.0 min; and compound 6, 47.0 min. The HPLC-DAD analyses of extracts showed that cyanidin 3-*O*-glucopyranoside 5 and free ellagic acid 6 are present only in achenes while compounds 2–4 were identified only in thalamus. As ellagic acid includes both free and conjugated forms, achenes and thalamus extracts were hydrolyzed in order to analyze ellagic acid more accurately. Again, ellagic acid was found only in achenes and gallic acid was identified in hydrolyzed thalamus extract.

Häkkinen et al. (34) reported that ellagic acid comprises 51% of the phenolic compounds in strawberries, and Hannum (26) has shown anthocyanins as the most abundant flavonoids in these berries. Our findings with the Chilean strawberry suggest a different aromatic compound (including phenolics) distribution

Table 4. Total Phenolic, Flavonoid Content, and Total Anthocyanins in Achenes, Thalamus, and Whole Fruit of *F. chiloensis*^a

	total phenolics ^b	total flavonoids ^c	total anthocyanins ^d
whole fruit	106.26 ± 3.50	29.96 ± 0.60	2.33 ± 0.91
achenes	4294.78 ± 26.18	2260.10 ± 55.40	104.25 ± 24.89
thalamus	73.06 ± 1.42	7.76 ± 0.04	0.213 ± 0.04

^aResults are presented as means ± SD. ^bQuantified as mg gallic acid equivalents/100 g FW. ^cQuantified as mg quercetin equivalents/100 g FW. ^dQuantified as mg cyanidin-3-O-β-D-glucopyranoside equivalents/100 g FW.

in *F. chiloensis*. Additional studies including more plant populations should be undertaken to confirm these findings.

The total phenolic, flavonoid, and anthocyanin contents in *F. chiloensis* as determined by spectrophotometry are presented in **Table 4**. The highest contents were found in the achenes. The w/w ratio between thalamus and achenes in *F. chiloensis* fruits was about 86:1. However, achenes contribute 40, 77, and 85% of the total phenolics, flavonoids, and anthocyanins, respectively, found in the whole fruits. The total phenolic content for the *F. chiloensis* whole fruit was lower than the values reported (159–289 mg catechin equivalents/100 g FW) by Cordenunsi et al. (35) for six different *F. × ananassa* cultivars. The total phenolic content reported by Proteggente et al. (36) for strawberry extracts was 330 mg gallic acid equivalents/100 g FW. Meyers et al. (8) reported total anthocyanins values in strawberries between 21.9 and 48.0 mg cyanidin-3-O-β-D-glucopyranoside equivalents/100 g FW for whole fruit of eight different cultivars. Values from 13 to 55 mg of pelargonidin-3-glucoside equivalents/100 g FW for whole fruit were reported for six different *F. ananassa* cultivars (35).

The flavonoid contents found in *F. chiloensis* whole fruit, thalamus, and achenes were 29.96, 7.76, and 2260.10 mg quercetin equivalents/100 g of FW, respectively. The flavonoid value found in *F. chiloensis* whole fruit was lower than those reported by Meyers et al. (8), which ranged between 46.2 and 72.0 mg catechin equivalents/100 g FW. In the present report, the difference found could not be explained solely on the different methodology used and is most probably inherent to *F. chiloensis*. In the Chilean strawberry investigated, the radical scavenging compounds are not only anthocyanins but also other phenolics that could not be fully identified during the present study due to the low amount and complex composition of the mixtures. Hydroxybenzoic and hydroxycinnamic acid derivatives have been reported for strawberries (37) as well as quercetin and kaempferol glucosides or conjugates (36). Further studies are necessary to fully disclose the phenolic and flavonoid composition of *F. chiloensis* ssp. *chiloensis*.

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