Bioassay-guided Isolation of Antioxidant and Cytoprotective Constituents from a Maqui Berry (*Aristotelia chilensis*) Dietary Supplement Ingredient as Markers for Qualitative and Quantitative Analysis

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Abstract

Bioassay-guided phytochemical investigation of a commercially available maqui berry (*Aristotelia chilensis*) extract used in botanical dietary supplement products led to the isolation of 16 compounds, including one phenolic molecule, **1**, discovered for the first time from a natural source, along with several known compounds, **2–16**, including three substances not reported previously in *A. chilensis*, **2**, **14**, and **15**. Each isolate was characterized by detailed analysis of NMR spectroscopic and HRESIMS data, and tested for their in vitro hydroxyl radical scavenging and quinone-reductase inducing biological activities. A sensitive and accurate LC-DAD-MS method for the quantitative determination of the occurrence of six bioactive compounds, **6**, **7**, **10**–**12**, and **14**, was developed and validated using maqui berry isolates purified in the course of this study as authentic standards. The method presented can be utilized for dereplication efforts in future natural product research projects, or to evaluate chemical markers for quality assurance and batch-to-batch standardization of this botanical dietary supplement component.

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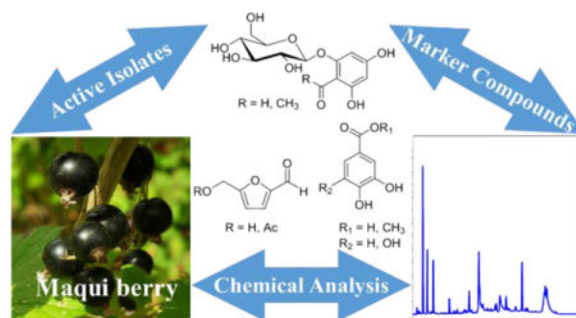
INTRODUCTION

According to the U.S. Centers for Disease Prevention, cancer is now the second leading cause of mortality in the United States, narrowly trailing the annual mortality attributed to heart disease. Cancer is already the most prevalent cause of deaths of Americans aged 45–64.[1] However, many authors have expressed the potential of reducing future cancer deaths, the disease burden, and the economic impact of oncotherapy by the chemoprevention of cancer initiation or progression.[2,3] In fact, several drugs have already been approved by the U.S. FDA for the purposeful prevention of some forms of cancers, although these agents have incompletely known mechanisms of action.[4,5] However, the induction of quinone reductase has been shown to correlate with other cytoprotective phase II enzymes, making it a suitable in vitro bioassay or "reasonable biomarker for the potential chemoprotective effect of test agents against cancer initiation."[6]

Among several health benefits correlated with the incorporation of berry fruits in the diet, positive impacts have been reported on various human health afflictions that include some forms of cancer.[7] The bioactive constituents in berries further have been shown to possess several possible roles in cancer chemoprevention, including protection against oxidative DNA damage by scavenging reactive oxygen species, enhancement of carcinogen detoxification processes via induction of phase II detoxifying enzymes, and modulation of signaling pathways involved with cellular inflammation, proliferation, apoptosis, angiogenesis and cell cycle arrest.[8] Additionally, the extractives from several berries have been validated in vivo as cancer chemopreventive agents against *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats, using a standardized protocol, providing additional evidence for the potential health benefits of berry consumption by humans.[9]

It has been reported that approximately one in six Americans used or had used non-vitamin non-mineral dietary supplements for the general improvement of their diet and health in the year 2012.[10] Indeed, the popularity of these products contributed to the observed increase in

financial scope of the U.S. dietary supplements market by ten-fold from an estimated $4 billion in 1994 to about $40 billion per year in 2014, marking just two decades since the Dietary Supplements Health and Education Act was passed by the U.S. Congress.[11] Included in this economic boom has been the new commercial availability of exotic and/or "super" fruit products, such as those derived from açaí, maqui berry, noni and pomegranate, to name a few. These ingredients are often perceived by consumers to have potent antioxidant capacities, and some have been studied scientifically in a quantitative and/or a qualitative fashion either as complex mixtures or through natural product isolation investigations.

One such dietary supplement ingredient is maqui berry [*Aristotelia chilensis* (Molina) Stuntz (Elaeocarpaceae)], the fruit of a South American evergreen shrub. The fruit itself is a small dark purple to black berry that can be eaten fresh or used in the preparation of jam, wine, and powdered extracts for consumption.[12] Furthermore, *A. chilensis* fruits and leaves have reported uses in folk medicine, "to treat a variety of ailments, including sore throat, kidney pains, ulcers, fever, hemorrhoids, inflammation, diarrhea, lesions, migraines, and scars".[12] Accordingly, several studies have investigated the in vitro or in vivo biological activities of *A. chilensis* extracts. For example, a methanolic extract of these fruits was shown to have in vitro antioxidant and in vivo murine cardioprotective activities.[13] Later, a polyphenolic-rich fraction of the methanolic extract of *A. chilensis* was shown to have in vivo antidiabetic activity in a hyperglycemic and obese mouse model.[14] The range of in vitro effects observed from maqui berry extracts, as well as the known phytochemical profile of these samples has been extensively reviewed recently.[15] However, it is important to recognize that these bioactivities typically have been attributed to anthocyanin and non-anthocyanin polyphenols present in the berries.[15,16] Accordingly, HPLC analysis has been previously used to show the presence of bioactive phytochemicals, especially focusing on anthocyanins in maqui berry extract or crude subfractions thereof.[16–22] There has only been one report of natural product isolation from *A. chilensis* fruits, in which the purification of unspecified "individual flavonoids, phenolic acids, anthocyanins and proanthocyanins" was performed, and the biological activity test results for these molecules were not disclosed.[23]

In the work presented herein, a maqui berry (*Aristotelia chilensis*) fruit extract used for formulation in dietary supplement products was studied by bioassay-guided fractionation to examine its potential cancer chemopreventive activity. This research yielded 16 pure compounds, including one molecule not previously isolated from any natural source, **1**, along with a closely related phloroglucinol glycoside, **2**, and two furanaldehydes, **14** and **15**, not previously detected in or isolated from maqui berries. Six phenolic compounds, **6**, **7**, **10**–**12**, and **14**, were selected as marker compounds and utilized as authentic standards for the development and validation of an LC-DAD-MS method, because they were relatively abundant in the sample and active in the in vitro hydroxyl radical-scavenging or quinone reductase- inducing bioassays. This analytical method was designed to be complementary to the work of others, described above, and allows for qualitative and quantitative analysis of the selected molecules in maqui berry extracts or other phytochemical samples. These results can thus be utilized for extract standardizations, quality assurance and quality control protocols, and dereplication efforts in natural product drug discovery.

MATERIALS AND METHODS

Instrumentation

Optical rotations were measured on a model 343 polarimeter (PerkinElmer, Waltham, MA) with a path length of 10 mm. A U-2910 spectrophotometer (Hitachi, Tokyo, Japan) was used to record UV spectra, and a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA) was used to obtain IR spectra. All NMR spectroscopic data were recorded on Avance DRX-400 and 600 MHz spectrometers (Bruker, Billerica, MA) operating at room temperature with standard built-in experiments. A Q-TOF II (Micromass, Wythenshawe, UK) mass spectrometer was operated in the positive-ion mode to obtain high-resolution electrospray ionization mass spectra (HRESIMS), and sodium iodide was used for mass calibration. Column chromatography was performed using Sephadex LH-20 (Supelco, Bellefonte, PA), normal phase silica gel of 65×250 or 230×400 mesh size (Sorbent Technologies, Atlanta, GA), and 40–63 μm particle size C_{18}-RP silica gel (Acros Organics, Geel, Belgium). Analytical thin-layer chromatography (TLC) was performed using pre-coated 250 μm thickness Partisil Si gel $60F_{254}$ glass plates, while preparative TLC was conducted on pre-coated 500 or 1000 μm thickness Partisil Si gel $60F_{254}$ glass plates (Whatman, Clifton, NJ). The column used for analytical HPLC was a 150 mm $\times$ 4.6 mm i.d., 5 μm, XBridge C_{18}, with a 10 mm $\times$ 4.6 mm i.d., 5 μm, guard column of the same material (Waters), while the column used for semi-preparative HPLC was a 150 mm $\times$ 10 mm i.d., 5 μm, Sunfire PrepC_{18}, with a 10 mm $\times$ 10 mm i.d., 5 μm, guard column of the same material (Waters). These columns were operated with a system (Hitachi, Tokyo, Japan) composed of an L-2130 prep pump, an L-2200 autosampler, and an L-2450 diode array detector.

Chemicals

All solvents used for chromatographic separations (ACS reagent, HPLC, and LC-MS grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin (BSA), 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA), digitonin, DMSO, EDTA, esterase, $FeSO_4$, flavin adenine dinucleotide phosphate (FAD), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P-D), H_2O_2, menadione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), nicotinamide adenine dinucleotide phosphate (NADP), quercetin, L-sulforaphane, Trizma base, and Tween 20, were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY). Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA).

Plant Material

Air-dried maqui berry (*A. chilensis*) fruit powder (lot # 09559; sample # 3410), originally collected in Chile, was obtained from Nature's Sunshine Products, Inc. (Spanish Fork, UT) for this study. A representative sample (OSUADK-CCP0024) has been deposited in the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University.

Extraction and Isolation

Milled dried fruits of *A. chilensis* (500 g) were repeatedly extracted by soaking with an excess of MeOH (3×1 L) at room temperature. The crude extract (ca. 181 g) was suspended in H_2O (1 L) and sequentially partitioned with hexanes (3×1 L), $CHCl_3$ (3×1 L), and *n*-BuOH (3×1 L) to afford hexanes (53.5 g), $CHCl_3$ (21.9 g), EtOAc (7.0 g), *n*-BuOH (10.0 g), and H_2O (ca. 89 g) partitions, respectively, after drying under vacuum. The EtOAc and *n*-BuOH partitions were both found to be active in the in vitro hydroxyl radical scavenging assay ($ED_{50} = 0.6$ and 1.0 μg/mL, respectively), and were subjected to chromatographic purification.

The EtOAc partition (D3) was first passed over a coarse silica gel open column (7.2 cm i.d.) with 280 g of coarse silica gel and eluted with a step gradient of $CHCl_3$/MeOH mixtures (40:1, 30:1, 20:1, 15:1, 8:1, 6:1, 4:1, 2:1, 1:1, 1:2, and 0:1). The eluent was collected in 250 mL aliquots that were combined, after analysis by TLC, to afford eight fractions (D3F01–D3F08). The *n*-BuOH partition (D4) was initially passed over an RP C_{18} open column using MeOH/H_2O mixtures (0:1, 1:4, 1:1, 4:1, and 1:0) as eluting solvents, to afford five fractions (D4F01–D4F05). Fractions showing activities in either or both of the hydroxyl radical-scavenging and quinone reductase-inducing assays were selected for further purification.

Fraction D3F02 (333 mg) was separated on a silica gel column with a solvent system of $CHCl_3$/MeOH (15:1, 10:1, 7:1, 3:1, and 1:1) to produce five subfractions (D3F0201–D3F0205). The subfraction D3F0202 was further refined by preparative TLC developed with $CHCl_3$/MeOH/AcOH (12:1:0.1), to yield compounds **14** ($R_f = 0.29$; 14.2 mg) and **15** ($R_f = 0.71$; 3.6 mg). Fraction D3F0205 was chromatographed on a Sephadex LH-20 column, with elution by MeOH/H_2O (1:1), to purify compound **16** (4.4 mg).

Fraction D3F03 (588 mg) was chromatographed over a silica gel column with a $CHCl_3$/MeOH solvent system (15:1, 10:1, 8:1, 5:1, and 2:1) to produce five subfractions (D3F03F01–D3F03F05). Subfraction D3F0303 was further purified by preparative TLC, developed using $CHCl_3$/MeOH/AcOH (7:1:0.1), to yield compounds **12** ($R_f = 0.20$; 16.8 mg) and **13** ($R_f = 0.66$; 2.2 mg). The fourth subfraction D3F0304 was further chromatographed over a Sephadex LH-20 column, with elution by MeOH/H_2O (40:60), to afford compounds **10** (19.8 mg), **11** (5.6 mg), and a further subfraction D3F030401. This subfraction D3F030401 was purified by semi-preparative RP-HPLC, using a CH_3CN/H_2O (35:65) isocratic elution at a flow rate of 4.0 mL/min, to yield compound **6** ($t_R = 20.1$ min; 8.2 mg).

Fraction D3F06 (416 mg) was subjected to Sephadex LH-20 column chromatography, with elution by MeOH/H_2O (1:3, 1:1, 3:1, and 1:0), to afford five subfractions (D3F0601–D3F0605). Of these subfractions, D3F0602 (eluted with MeOH/H_2O, 50:50) was further purified by RP-HPLC with isocratic elution (MeOH/H_2O, 3:7), at a flow rate of 4.0 mL/min, to yield compounds **1** ($t_R = 25.2$ min; 1.6 mg) and **2** ($t_R = 32.7$ min; 3.8 mg). In addition, D3F0603 (eluted with MeOH/H_2O, 75:25) was also purified by RP-HPLC, with isocratic elution (CH_3CN/H_2O, 26:74) at a flow rate of 4.0 mL/min, to yield compounds **7** ($t_R = 22.8$ min; 4.1 mg), **8** ($t_R = 31.3$ min; 2.1 mg), and **9** ($t_R = 36.5$ min; 3.0 mg).

Fraction D4F02 (669 mg) was purified initially over a Sephadex LH-20 column, with elution by a MeOH/H$_2$O step-gradient (0:1, 1:3, 1:1, 3:1, and 1:0), to afford five subfractions (D4F0201–D4F0205). Subfraction D4F0202 was then separated by RP-HPLC with a MeOH/H$_2$O gradient (flow rate 4.0 mL/min, 20–50% MeOH with 0.05% TFA from 0 to 60 min), to yield compounds **3** (t_R = 42.6 min; 11.9 mg), **4** (t_R = 30.3 min; 7.2 mg), and **5** (t_R = 18.5 min; 5.8 mg).

2-O-β-D-Glucopyranosyl-4,6-dihydroxybenzaldehyde (1)—isolated (ca. 0.0003% of dry weight) as a colorless resin; [α]^{20}$_D$ –78 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 219.5 (4.30), 285.0 (4.33) nm; IR (film) ν_{max} 3333, 2919, 1642, 1608, 1524, 1466, 1446, 1379, 1315, 1267, 1222, 1166, 1080, 895 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); HRESIMS *m/z* 339.0679 [M+Na]$^+$ (calcd for C$_{13}$H$_{16}$O$_9$Na, 339.0687).

Evaluation of in vitro Hydroxyl Radical-scavenging Activity

A hydroxyl radical-scavenging activity bioassay was performed according to a method described previously.[24–26]

Evaluation of in vitro Quinone Reductase (QR)-inducing and Cytotoxicity Activities

The potential QR-inducing activity and cytotoxicity of the extracts, fractions, and pure isolates was assayed in vitro using murine Hepa1c1c7 cells, as described previously.[25,27]

Optimized Extraction Procedure

To complement previous reports on the determination of anthocyanins in *A. chilensis* fruits,[16–23] the present study focused on the determination of other major phenolic and flavonoid constituents that were shown to be active in the hydroxyl radical-scavenging and QR-inducing assays. Thus, on the basis of previously reported extraction methods for phenolic and flavonoid components,[28,29] with minor modification, the extraction method was optimized and further confirmed by an orthogonal test. Briefly, 2 g of milled dry fruits of *A. chilensis* were accurately weighed and extracted with 10 mL of MeOH/H$_2$O (95:5) by sonication at room temperature for 15 min. After vacuum filtration, the residue was extracted with 10 mL EtOH/H$_2$O (80:20) by sonication at ambient temperature for 15 min, followed by filtration. Both filtrates were combined and dried under vacuum at 40 °C, then re-dissolved in 200 μL of MeOH. This MeOH extract was passed over a Waters Sep-Pak Vac 20cc C$_{18}$ cartridge to remove the very polar substances present (eluted by MeOH/H$_2$O, 5:95), followed by elution with MeOH/H$_2$O (95:5) to afford the pre-treated extract. This pre-treated extract was dried and transferred into a 25 mL volumetric flask and brought up to the full volume in MeOH to prepare the extract solution for HPLC-DAD and LC-MS analysis.

HPLC-DAD Separation and Analysis

The HPLC-DAD analysis was performed using a 150 mm × 4.6 mm i.d., 5 μm, XBridge C$_{18}$ column, with a 10 mm × 4.6 mm i.d., 5 μm, guard column of the same material (Waters) in a system composed of an L-2130 pump and an L-2450 diode array detector (Hitachi). The mobile phase consisted of 0.05% TFA in H$_2$O (A) and CH$_3$CN (B), using a gradient program of 5–20% B from 0 to 60 min and 20–100% B from 60 to 70 min. The mobile

phase flow rate and the injection volume were 1 mL/min and 10 μL, respectively. The column temperature was set at 30 °C, and the chromatograms were recorded at wavelengths from 200–550 nm.

LC-IT-MS Analysis

The LC-IT-MS analysis procedure employed the same separation conditions as used for the HPLC-DAD analysis mentioned above, on an Alliance 2690 Separation Module (Waters) using the same XBridge C_{18} analytical column as above (Waters). The injection volume and mobile phase flow rate were 10 μL and 1 mL/min, respectively. Approximately 2% of the column eluent was split to the MS using a microsplitter valve 203 (Upchurch Scientific, Oak Harbor, WA). A dual funnel amaZon ETD Ion Trap mass spectrometer (Bruker, Bremen, Germany) equipped with an orthogonal electrospray source was used for electrospray ionization ion trap mass spectrometry (ESI-IT-MS), and this was operated in positive-ion mode with sodium iodide being used for mass calibration in the range of m/z 100–1000. The optimal ESI conditions used were: capillary voltage 4500 V, source temperature 250 °C, N_2 was used as the ESI drying gas at 4.0 L/min and as the nebulizer gas at 10 psi. The ion trap was set to UltraScan mode with a target mass of m/z 500 pass ions from m/z 100–1000.

Quantitation of the Major Bioactive Constituents of *A. chilensis* Fruits

Stock solutions of the six standards (compounds **6**, **7**, **10**–**12**, and **14**) were prepared in MeOH, each at 2500 mg/L. Work solutions were obtained as a mixture of these stock solutions after series dilutions with methanol to achieve five concentration levels in the range of 2–250 mg/L. The work solutions were filtered through a 13 mm, 0.2 μm pore size, syringe filter (Fisher Scientific, Fair Lawn, NJ) prior to HPLC injection. The linearity was plotted using linear regression analysis by the integrated peak areas (*Y*) vs. concentration of each standard (*X*, mg/L) at five different concentrations.

Validation of the Analysis

The validation for limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, and recovery was implemented under the present analytical procedures.[30] The LOD and LOQ were determined at the signal-to-noise ratio of 3:1 and 10:1, respectively. The inter-day and intra-day precisions were evaluated by relative standard deviation (RSD) under six repeated injections using work standard solution within 1 d and 3 d, respectively. The repeatability was assessed by extracting and analyzing the same batch of *A. chilensis* fruit powder six times, independently from each other. Recovery tests were carried by spiking an amount of standards with known content to the same batch of samples to calculate the ratio of the detected and added samples.

Statistical Analysis

All experimental data of the hydroxyl radical-scavenging and quinone reductase-inducing activities were determined as means ± standard deviations (SD).

RESULTS AND DISCUSSION

Bioactivity-guided Isolation of Chemical Constituents

Among the hexanes, CHCl$_3$, EtOAc, n-BuOH, and H$_2$O partitions of the *A. chilensis* fruit extract, the EtOAc (D3) and n-BuOH (D4) partitions were found to be most potent in the in vitro hydroxyl radical-scavenging assay (ED$_{50}$, concentration scavenging hydroxyl radicals by 50% = 0.6 and 1.0 μg/mL, respectively), so therefore these two samples were selected for further fractionation. Afterward, subfractions D3F03, D3F06, and D4F02 showed hydroxyl radical-scavenging activity with ED$_{50}$ = 0.5, 0.4, and 0.3 μg/mL, respectively. Additionally, fractions D3F02 and D3F03 exhibited quinone reductase (QR)-inducing activity (CD, concentration doubling QR activity = 19.5 and 9.6 μg/mL, respectively). Accordingly, these fractions were subjected to further purification steps. In this way, the bioactivity-guided fractionation of *A. chilensis* fruit extract led to the isolation of a new natural product, **1**, together with fifteen known compounds, **2**–**16**. As shown in Figure 1, this set of molecules comprises twelve phenolic compounds, **1**–**13**, two furan derivatives, **14** and **15**, and an organic acid, **16**. Compounds **6**, **7**, **10**–**12**, and **14** were found to be the major non-anthocyanin constituents. Furthermore, this is the first time that the phloroglucinol glycosides, **1** and **2**, and furan compounds, **14** and **15**, were reported to be present in *A. chilensis* fruits.

Structure Elucidation of the Purified Natural Product 1

The new compound **1** was obtained as a colorless resin. The molecular formula of **1** was determined as C$_{13}$H$_{16}$O$_9$ on the basis of the sodiated molecular ion peak in the HRESIMS. The IR spectrum exhibited absorptions of hydroxy (3333 cm^{-1}), conjugated carbonyl (1642 cm^{-1}), and phenyl groups (1608, 1524, and 1446 cm^{-1}). The UV spectrum showed a peak at 285 nm typical of an aryl conjugated carbonyl, such as the chromophore of a phloroglucinol glycoside.[31] The ^{1}H NMR spectrum collected in CD$_3$OD exhibited a downfield signal at δ_H 10.11 (1H, s, CH-7), which was further confirmed by the observation of a downfield carbon at δ_C 193.2, indicating the presence of a formyl group. Additionally, a pair of weak-coupled downfield signals in the ^{1}H-^{1}H COSY spectrum suggested the presence of an isolated 1,2,3,5-tetrasubstituted aromatic AX spin system at δ_H 6.17 (1H, d, J = 2.2 Hz; δ_C 95.9, CH-3) and 5.91 (1H, d, J = 2.2 Hz; δ_C 100.0, CH-5). A second isolated spin system, observed in the mid-field region of the ^{1}H NMR spectrum, was especially characteristic of a glucosyl moiety on the basis of the observation of appropriately deshielded carbons in the ^{13}C NMR spectrum { δ_H 4.96 (1H, d, J = 7.4 Hz; δ_C 102.1, CH-1$'$), [3.91 (1H, dd, J = 12.0, 1.8 Hz) and 3.72 (1H, dd, J = 12.0, 4.9 Hz); δ_C 62.3, CH$_2$-6$'$], 3.50 (1H, t; δ_C 74.7, CH-2$'$), 3.46 (1H, m; δ_C 78.4, CH-3$'$), 3.46 (1H, m; δ_C 77.9, CH-5$'$), and 3.41 (1H, m; δ_C 71.1, CH-4$'$)}. The coupling constant of 7.4 Hz observed for the anomeric proton (H-1$'$) indicated a β-configuration of the glucose linkage. The connection of the three structural subunits was determined based on key correlations from the ^{1}H-^{13}C HMBC spectrum (Figure 2). For example, the oxygenated aromatic carbon at δ_C 163.5 (C-2) was determined to be the point of glycosylic substitution, because it showed an HMBC correlation with the anomeric (H-1$'$) proton. Furthermore, one aromatic proton (H-3) and the formyl (H-7) proton also both had HMBC correlations to C-2. Another oxygenated aromatic carbon, at δ_C 169.3 (C-6), had an HMBC correlation with the formyl proton and the other aromatic proton

in the molecule (H-5). The last oxygenated aromatic carbon was observed at δ_C 166.8 (C-4), which had HMBC correlations only with H-3 and H-5. Finally, both of the aromatic protons (H-3 and H-5) and the formyl proton (H-7) all exhibited correlations in the HMBC spectrum to a relatively upfield non-protonated aromatic carbon at δ_C 106.8 (C-1), corresponding to the point of connection of the formyl group to the aromatic ring, and thus concluding the planar structure of **1**. The ^{1}H NMR spectrum for this molecule was found to be closely comparable to that of a second substance isolated in the course of this investigation, compound **2** (Table 1). The identity of compound **2** was determined as 4,6-dihydroxy-2-*O*-(*β*-D-glucopyranosyl)acetophenone by comparison of its physical data and chromatographic behavior with an authentic standard.[32] Since the specific rotation values of **1** and **2** were consistent with each other {$[\alpha]^{20}_D$ –78 and –84 (*c* 0.1, MeOH), respectively}, the absolute configuration was assigned to match, which established **1** as 2-*O*-*β*-D-glucopyranosyl-4,6-dihydroxybenzaldehyde. The structure of **1** was previously reported, without characterization data for comparison, as a synthetic intermediate in the generation of anthocyanin.[33]

In addition to **1** and **2**, the identities of another 14 isolated compounds were determined by comparison of their spectroscopic, spectrometric, and specific rotation data with literature values. These were established as being cyanidin 3-*O*-*β*-D-glucopyranoside, **3**,[34] delphinidin 3-*O*-*β*-D-glucopyranoside, **4**,[34] cyanidin 3-*O*-*β*-D-sambubioside, **5**,[35] caryatin, **6**,[36] hyperoside, **7**,[37] quercetin 3-*O*-*a*-L-arabinofuranoside, **8**,[37] quercetin 3-*O*-*β*-D-xylopyranoside, **9**,[37] gallic acid, **10**,[38] gallic acid methyl ester, **11**,[39] protocatechuic acid, **12**,[32] protocatechuic acid methyl ester, **13**,[40] hydroxymethylfurfural, **14**,[41] acetyloxymethylfurfural, **15**,[42] and 1,5-dimethyl citrate, **16**.[43]

Hydroxyl-radical Scavenging and Quinone Reductase-inducing Activities of the Pure Compounds

Although previous studies have linked the phenolic and anthocyanin fractions of *A. chilensis* fruits to their observed high antioxidant activity,[21,22] no pure constituents of *A. chilensis* have been isolated for biological evaluation and reported previously. Thus, all sixteen pure compounds isolated in the present investigation were evaluated for their hydroxyl radical-scavenging and quinone reductase induction activities using standard procedures. As shown in Table 2, except for **15** and **16**, which showed activity in neither of these two assays, compounds **1**–**14** were all found to be active in one or both of the tests. Among them, compounds **1**–**13** showed demonstrable hydroxyl radical-scavenging activity, with cyanidin 3-*O*-*β*-D-glucopyranoside, **3** (ED_{50} = 0.13 μM), caryatin, **6** (ED_{50} = 0.18 μM), and hyperoside, **7** (ED_{50} = 0.17 μM) exhibiting the greatest potencies. Remarkably, the majority of these compounds, **3**–**11**, each showed a higher potency than the positive control, quercetin, which is known to be a quite potent hydroxyl radical scavenger. In addition, four compounds, **7**, **11**, **12**, and **14**, exhibited quinone reductase-inducing activity, with protocatechuic acid, **12** (CD = 4.1 μM) being the most potent.

Utilization of the Major Bioactive Non-anthocyanin Phenolics as Markers for Quantitative Analysis

Since previous phytochemical analysis of *A. chilensis* fruits have focused on anthocyanins and have established some HPLC methods for quantitation of several major anthocyanin constituents,[16–22] the present study aimed to investigate the chemical profile and biological activity of the non-anthocyanin phenolics of *A. chilensis* fruits. Although the total phenolic contents of *A. chilensis* fruits have been studied and linked to antioxidant activity,[17,21,23] the composition of these components has not yet been quantitated. As mentioned above, some of these non-anthocyanin phenolics were found to be present in *A. chilensis* fruits in high quantities during the isolation process and showed direct hydroxyl-radical scavenging and quinone reductase-inducing activities in the assays. Thus, the major bioactive non-anthocyanin phenolics **6**, **7**, and **10**–**12** along with the major bioactive furan compound **14**, which has not been previously identified from *A. chilensis*, were selected as markers for the development of a quantitative analysis method to reflect simultaneously the chemical profile and biological activity of *A. chilensis* fruits.

Quantitative Analysis of the Major Bioactive Non-anthocyanin Components

An HPLC–DAD–MS analysis method, a proven effective tool for botanical dietary supplement identification,[44] was developed to achieve good separation and detection of the selected major chemical constituents of *A. chilensis* fruits, with a representative chromatogram shown in Figure 3. By comparing the retention times, UV profiles, and [M +H]$^+$ and [M+Na]$^+$ molecular ions of the peaks with those of authentic standards (Table 3), the six selected major bioactive non-anthocyanin components could be identified unambiguously as gallic acid, **10**, hydroxymethylfurfural, **14**, protocatechuic acid, **12**, gallic acid methyl ester, **11**, hyperoside, **7**, and caryatin, **6**, respectively. After comparing the chromatograms of the extract solution recorded at wavelengths within 200–550 nm, it was found that 300 nm could best represent the profile of the analytes. At this observation wavelength, a good linearity was achieved for each of the quantitated constituents (R^2 >0.999), and the contents of these constituents were determined using the developed quantitative method (Table 4).

Validation

A comprehensive validation of the quantitative method described above was conducted. The LOD and LOQ were in suitable low ng ranges for the major bioactive compounds to be analyzed. The values of RSD for the precision, repeatability, and recovery tests were all <3%. The validation data for quantitation of the selected major bioactive constituents of *A. chilensis* are shown in Table 5. Altogether, these results indicated that the method was satisfactory for sample analysis.

In the work presented herein, a maqui berry (*Aristotelia chilensis*) fruit extract, used as a dietary supplement ingredient, was investigated by bioassay-guided fractionation. This yielded 16 natural product isolates, including one molecule not previously disclosed from any natural source, **1**, together with a closely related phloroglucinol glycoside, **2**, and two furanaldehydes, **14** and **15**, that were never before reported or detected in maqui berry samples. All isolates, **1**–**16**, were evaluated for potential cancer chemopreventive activity

using hydroxyl-radical scavenging and quinone reductase-induction bioassays. Six bioactive phenolic compounds, **6**, **7**, **10**–**12**, and **14**, were selected as marker compounds and utilized as authentic standards for the development and validation of an LC-DAD-MS method because they were present in high abundance, demonstrated in vitro bioactivities, and were not previously used in existing methods designed for analysis of maqui berry samples. Since this analytical method allows for qualitative and quantitative determination of the analytes in maqui berry extracts or other phytochemical samples, it can be utilized for extract standardizations, quality assurance and control protocols, and dereplication efforts in natural product drug discovery. This study has thus provided three of the recommended components of proposed best practices in compliance with the FDA good manufacturing practice (GMP) guidelines intended to produce consistent, effective and safe botanical dietary supplements, notably the identification of bioactive constituents of the botanical of interest, their development as chemical markers for chemical standardization, and the use of in vitro bioassays pertinent to cancer chemoprevention as preliminary biological standards.[45,46] These results also represent the first direct evidence that partially correlate the reported beneficial health effects of *A. chilensis* fruit consumption to the identified chemical constituents, and indicate that *A. chilensis* may be of interest for further investigation of cancer chemopreventive agents or as a possible natural functional food ingredient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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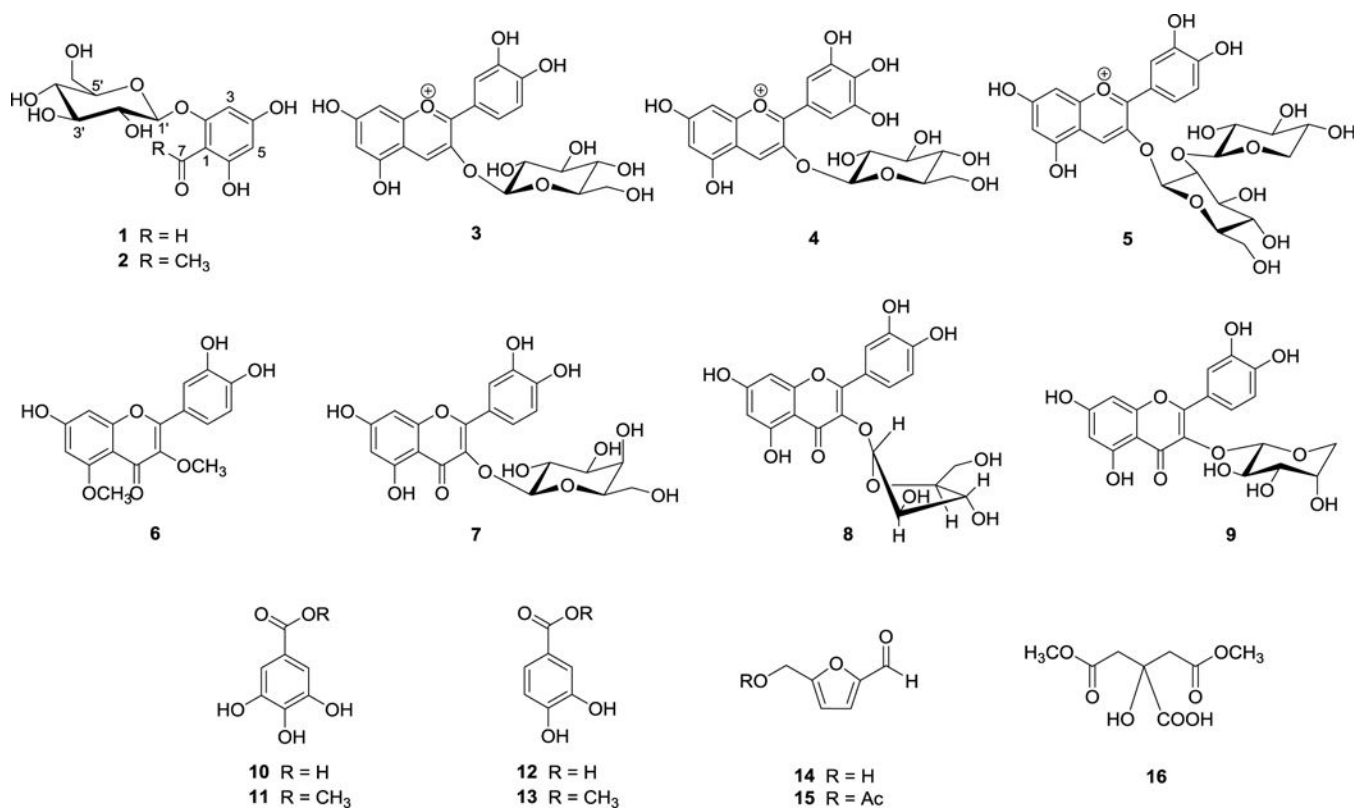
Figure 1.
Structures of compounds isolated from the fruits of maqui berry (*A. chilensis*).
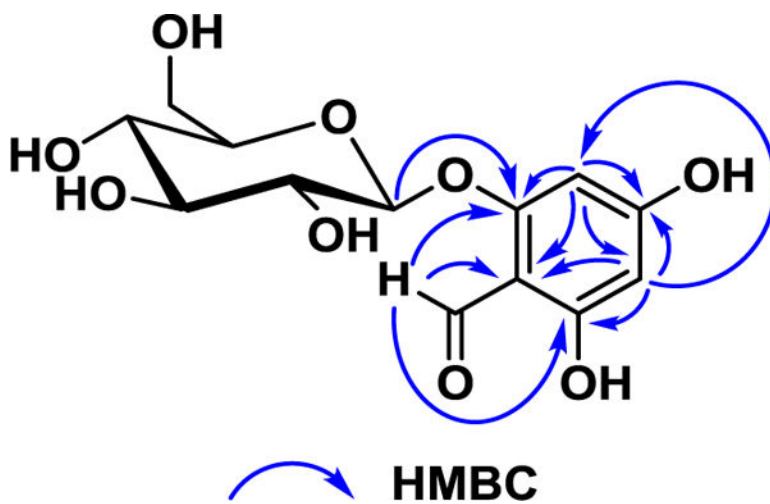
Figure 2.
Key HMBC correlations of the new natural product **1** isolated from maqui berry.
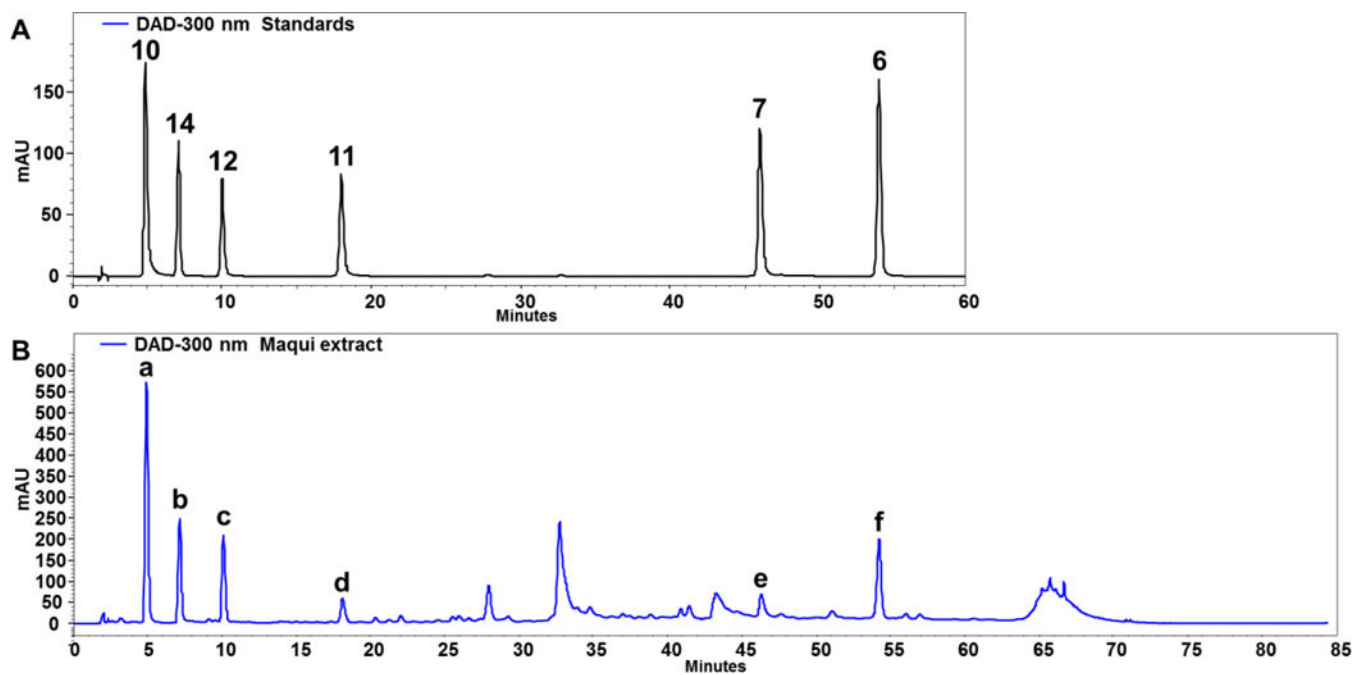
Figure 3.
A representative HPLC chromatogram of chemical constituents of maqui berry (*A. chilensis*). (A). Major bioactive non-anthocyanin components isolated as standards with bold compound numbers corresponding to structures presented in Figure 1. (B). Maqui berry fruit extract with identified peaks labeled **a**-**f** described in Table 3.

Table 1

Comparison of the ^{1}H (mult., J in Hz) and ^{13}C NMR Spectroscopic Data of Compounds **1** and **2** in CD$_3$OD.^{a,b,c}

| position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	–	106.8	–	106.7
2	–	163.5	–	162.6
3	6.17 (d, 2.2)	95.9	6.18 (d, 2.2)	95.4
4	–	166.8	–	166.3
5	5.91 (d, 2.2)	100.0	5.94 (d, 2.2)	98.1
6	–	169.3	–	167.7
7	10.11 (s)	193.2	–	204.8
1′	4.96 (d, 7.4)	102.1	5.02 (d, 7.4)	102.0
2′	3.50 (t, 7.4)	74.7	3.54 (t, 7.4)	74.7
3′	3.46 (m)	78.4	3.46 (m)	78.5
4′	3.41 (m)	71.1	3.42 (m)	71.1
5′	3.46 (m)	77.9	3.46 (m)	78.3
6′	3.91, (dd, 12.0, 1.8)	62.3	3.92, (dd, 12.0, 1.8)	62.4
	3.72, (dd, 12.0, 4.9)		3.72, (dd, 12.0, 4.9)	
CH$_3$	–	–	2.69 (s)	33.5

^{a}Measured at 400 MHz for ^{1}H NMR with residual signals of CD$_3$OD at δ 3.31 ppm used as reference.

^{b}Measured at 100 MHz for ^{13}C NMR with residual signals of CD$_3$OD at δ 49.0 ppm used as reference.

^{c}Assignments supported by 2D NMR experiments.

Table 2

Hydroxyl Radical-Scavenging and Quinone Reductase-Inducing Activities of the Isolated Compounds, **1 – 16**, from the Fruits of Maqui Berry (*A. chilensis*).

compound	hydroxyl radical scavenging $ED_{50}{}^a$ (μM)	quinone reductase (QR) induction		
		CD^b (μM)	$IC_{50}{}^c$ (μM)	CI^d
1	1.3 ± 0.12	>20	>100	N/A^e
2	1.4 ± 0.16	>20	>100	N/A
3	0.13 ± 0.02	>20	>100	N/A
4	0.64 ± 0.06	>20	>100	N/A
5	0.69 ± 0.08	>20	>100	N/A
6	0.18 ± 0.02	>20	>100	N/A
7	0.17 ± 0.02	19.2 ± 2.8	>100	>5.2
8	0.46 ± 0.05	>20	>100	N/A
9	0.46 ± 0.04	>20	>100	N/A
10	0.41 ± 0.05	>20	55.3 ± 7.8	N/A
11	0.33 ± 0.04	14.1 ± 2.3	82.4 ± 11.9	5.8 ± 0.82
12	1.9 ± 0.21	4.1 ± 0.78	>100	>24.4
13	1.8 ± 0.16	>20	>100	N/A
14	>20	18.7 ± 2.9	>100	>5.1
15	>20	>20	>100	N/A
16	>20	>20	>100	N/A
quercetin^f	1.2 ± 0.11			
L-sulforaphane^g		0.77 ± 0.11	16.7 ± 1.9	21.7 ± 2.8

^{a}ED$_{50}$, concentration reducing scavenging hydroxyl radicals by 50%. Each value represents the mean ± SD (n = 3). Compounds with ED$_{50}$ values of <20 μM are considered active.

^{b}CD, concentration required to double quinone reductase activity. Each value represents the mean ± SD (n = 3). Compounds with CD values of <20 μM are considered active.

^{c}IC$_{50}$, concentration inhibiting cell growth by 50%.

^{d}CI, chemoprevention index (= IC$_{50}$/CD).

^{e}NA, not applicable.

^{f}Positive control for hydroxyl radical-scavenging assay.

^{g}Positive control for quinone reductase-induction assay.

Table 3

LC-MS Data of the Major Bioactive Constituents Identified in Maqui Berry (*A. chilensis*) Extract.

peak	t_R (min)	UV λ_{max} (nm)	MF	$[M+H]^+$	$[M+Na]^+$	identification
a	5.0	216, 272	$C_7H_6O_5$	171.1	193.1	gallic acid (**10**)
b	7.1	229, 284	$C_6H_6O_3$	127.1	149.1	hydroxymethylfurfural (**14**)
c	10.1	218, 260, 294	$C_7H_6O_4$	155.1	177.1	protocatechuic acid (**12**)
d	18.0	216, 272	$C_8H_8O_5$	185.1	207.1	gallic acid methyl ester (**11**)
e	46.1	255, 355	$C_{21}H_{20}O_{12}$	465.1	487.1	hyperoside (**7**)
f	54.1	252, 348	$C_{17}H_{14}O_7$	331.1	353.1	3,5-dimethoxyquercetin (**6**)

Table 4

Quantitative Analysis of the Major Bioactive Constituents in Maqui Berry (*A. chilensis*) Fruits (per dry weight).

constituent	retention time (min)	regression equation[a] (Y = aX + b, R²)	linear range (mg/L)	content[b] (mg/kg)
10	5.0	$Y = 33283X + 3049, 0.9996$	2–250	202 ± 1.1
14	7.1	$Y = 66525X + 544211, 0.9993$	2–250	98 ± 0.6
12	10.1	$Y = 23960X + 121915, 0.9995$	2–250	108 ± 0.6
11	18.0	$Y = 22844X + 229491, 0.9993$	2–250	28 ± 0.2
7	46.1	$Y = 35226X + 115364, 0.9996$	2–250	20 ± 0.1
6	54.1	$Y= 58063X + 139757, 0.9998$	2–250	68 ± 0.4

[a]Y = peak area and X = concentration.

[b]Mean ± SD (n = 3).

Table 5

Method Validation (Limits of Detection, Accuracy, and Precision) for Quantitation of the Major Bioactive Constituents of Maqui Berry (*A. chilensis*) Fruits.

constituent	LOD (ng)	LOQ (ng)	precision (RSD, n = 6) intra-day	precision (RSD, n = 6) inter-day	repeatability (n = 6) RSD	recovery (n = 3) mean	recovery (n = 3) RSD
10	2.2	7.5	0.98%	2.17%	2.28%	92.06%	2.84%
14	2.0	6.7	0.88%	2.38%	1.82%	93.08%	2.99%
12	2.1	7.1	1.11%	2.21%	1.62%	90.32%	1.96%
11	2.4	7.8	0.86%	2.03%	1.42%	95.20%	2.54%
7	3.5	9.9	0.69%	1.98%	1.95%	91.49%	2.19%
6	2.9	9.1	0.77%	1.66%	1.11%	92.29%	1.88%