Quinoa seeds leach phytoecdysteroids and other compounds with anti-diabetic properties

Brittany L. Graf[1], **Alexander Poulev**[1], **Peter Kuhn**[1], **Mary H. Grace**[2], **Mary Ann Lila**[2], and **Ilya Raskin**[1]

[1]Department of Plant Biology & Pathology, Rutgers University, 59 Dudley Rd, New Brunswick, NJ, USA 08901

[2]Plants for Human Health Institute, North Carolina State University, 600 Laureate Way, Kannapolis, NC, USA 28081

Abstract

Quinoa (*Chenopodium quinoa* Willd.) contains high levels of biologically active phytoecdysteroids, which have been implicated in plant defense from insects, and have shown a range of beneficial pharmacological effects in mammals. We demonstrated that the most prevalent phytoecdysteroid, 20-hydroxyecdysone (20HE), was secreted (leached) from intact quinoa seeds into water during the initial stages of seed germination. Leaching efficiency was optimized by ethanol concentration (70% ethanol), temperature (80°C), time (4 h), and solvent ratio (5 ml/g seed). When compared to extraction of macerated seeds, the leaching procedure released essentially all the 20HE available in the seeds (491 μg/g seed). The optimized quinoa leachate (QL), containing 0.86% 20HE, 1.00% total phytoecdysteroids, 2.59% flavonoid glycosides, 11.9% oil, and 20.4% protein, significantly lowered fasting blood glucose in obese, hyperglycemic mice. Leaching effectively releases and concentrates bioactive phytochemicals from quinoa seeds, providing an efficient means to produce a food-grade mixture that may be useful for anti-diabetic applications.

Keywords

ecdysterone; flavonoids; metabolic syndrome; nutraceutical; phytoecdysteroids

1. Introduction

Quinoa (*Chenopodium quinoa* Willd., Amaranthaceae), an ancient crop from the Andes Mountains of South America, is rapidly gaining popularity as a functional food and nutraceutical (Vega-Galvez, Miranda, Vergara, Uribe, Puente, & Martinez, 2010). Due to

quinoa's nutritious and stress tolerant properties, the Food and Agriculture Organization of the United Nations launched the International Year of Quinoa in 2013 to promote the worldwide production, consumption, technological development and biodiversity preservation of this crop (FAO, 2012). The focus of quinoa's health benefits in scientific literature has primarily centered upon its macro- and micro-nutrient profile, especially its complete amino acid content. However, little work has been done to investigate the role of quinoa's secondary metabolites in human health.

Among agricultural food crops that are regularly sold and consumed in the United States, quinoa is a rich source of phytoecdysteroids (Kumpun, Maria, Crouzet, Evrard-Todeschi, Girault, & Lafont, 2011; Zhu, et al., 2001a). Phytoecdysteroids are polyhydroxylated steroids, structurally related to insect molting hormones, that have been implicated in plant defense by deterring insect herbivory, delaying insect development and causing lethality to insect larvae (Arnault & Slama, 1986; Blackford & Dinan, 1997; Marion-Poll & Descoins, 2002; Rharrabe, Sayan, & Lafont, 2010; Singh & Russell, 1980). Phytoecdysteroids have also shown a wide range of therapeutic effects in mammals, including anabolic, performance enhancing (Gorelick-Feldman, MacLean, Ilic, Poulev, Lila, Cheng, & Raskin, 2008; Slama & Lafont, 1995), anti-osteoporotic (Kapur, Wuttke, Jarry, & Seidlova-Wuttke, 2010; Seidlova-Wuttke, Christel, Kapur, Nguyen, Jarry, & Wuttke, 2010) and wound healing properties (Syrov & Khushbaktova, 1996). These molecules are considered the primary bioactive components of the traditional Chinese and Siberian herbs *Ajuga turkestanica*, *Rhaponticum carthamoides,* and *Cyanotis vaga* (Gorelick-Feldman, et al., 2008; Kokoska & Janovska, 2009; Lafont, 1998). Phytoecdysteroids, extracted from these botanical sources, have been marketed and sold in commercially available health products, such as adaptogens, body building agents, stress reducers, performance enhancers and cosmetics (Bathori, 2002). Recent literature also suggests that the most prevalent phytoecdysteroid, 20-hydroxyecdysone (20HE, also known as ecdysterone or β-ecdysone) may play a role in the treatment and prevention of diabetes and obesity. Administration of 20HE (10 mg/kg body weight for 13 weeks) to diet-induced obese, hyperglycemic mice significantly lowered blood glucose levels, increased insulin sensitivity, decreased body weight and reduced adiposity 41% compared with the control without affecting food consumption (Kizelsztein, Govorko, Komarnytsky, Evans, Wang, Cefalu, & Raskin, 2009).

Diabetes is a metabolic disorder that has reached epidemic proportions worldwide, especially among low and middle-income countries and communities, thereby demanding effective, sustainable and affordable intervention strategies aimed at glycaemic control (Zimmet, Magliano, Herman, & Shaw, 2014). While slow growing, wildcrafted perennial herbs have traditionally been harvested as a source of phytoecdysteroids, cultivated annual edible plants can provide a renewable source of these hypoglycemic molecules. Furthermore, a rapid, efficient, food-grade procedure for concentrating phytoecdysteroids from edible sources may facilitate the development of cost-effective therapeutics that can be integrated into the diet.

The highest concentration of 20HE previously reported in an extract of an edible crop was achieved through a multi-step solvent extraction of spinach leaves (*Spinacia oleracea* L.) (Gorelick-Feldman, et al., 2008). Quinoa seeds, however, contain 4 – 12 times more 20HE

by dry weight (184 – 484 μg/g) (Kumpun et. al., 2011) than spinach leaves (40 μg/g) (Gorelick-Feldman, et al., 2008), and are therefore more attractive for the development of 20HE-enriched botanicals. The elimination of hazardous organic solvents from the extraction procedure is also important for the development of food-grade technologies.

Foucault et al. (2011) has previously reported a method for producing a conventional 20HE-enriched extract (1.5 – 2.0% 20HE w/w) from quinoa seeds. However, this method resulted in a relatively low yield (10 mg extract/g seeds) and necessitated pulverization of the seeds upon boiling, followed by several extractions and drying steps.

We aimed to develop a one-step, non-destructive, high-yield process for concentrating phytoecdysteroids from quinoa seeds. To do so, we investigated the phenomenon of leaching of phytoecdysteroids from intact seeds into solution. We hypothesized that, if phytoecdysteroids play a role in the defense of quinoa seeds from insect herbivory, the molecules would leach from intact seeds into an aqueous environment during the initial stages of seedling germination. Therefore, the maximum secretion of phytoecdysteroids from seeds into water at environmental temperatures was measured, and optimized leaching using ethanol and heat. Furthermore, following on from earlier reports of the anti-diabetic properties of 20HE, we investigated the hypoglycemic activity of quinoa leachate (QL), in a high fat diet-induced, hyperglycemic C57Bl/6J mouse model.

2. Materials and Methods

2.1. Materials

Bolivian-grown red quinoa seeds were purchased from AlterEco, Inc. (San Francisco, CA), containing 11% protein, 10% carbohydrate and 4% fat (Nutrition Facts, AlterEco Red Quinoa). Seeds were polished and washed by the producer to remove the outer layer of bitter saponins before distribution. All chemical reagents were obtained from Sigma (St. Louis, MO), unless otherwise specified. All solvents used for compound isolation were HPLC grade. All water used in the experiments was purified using a Millipore water purification system with a minimum resistivity of 18.2 MΩ cm (Bedford, MA).

2.2. Aqueous leaching of 20HE

To prevent bacterial and fungal contamination, quinoa seeds were first surface-sterilized by immersion in 70% ethanol for 1 minute, followed by 12 minutes in 1.5% sodium hypochlorite, and rinsed three times with sterile water. Seeds were dried overnight on sterile filter paper. Seeds ($\approx$0.25 g) were incubated in 1 ml sterile ddH$_2$O in culture tubes for 24, 48, 72, 96 or 144 h (three replicates per time point) on a shaker at 160 rpm/ min in light and dark conditions at 25 or 37°C. We considered the effect of light versus dark conditions on 20HE leaching due to the possibility that quinoa seeds may synthesize 20HE during the germination process. 20HE leaching was monitored at room temperature (25°C) and elevated temperature (37°C), since heat is an important factor in chemical stability, solubility and seed germination rates. At the end of each incubation time point, the leachate from each sample was filtered through a 0.45 μm syringe filter (Corning, Inc., Corning, NY) into pre-weighed Eppendorf tubes and dried by speed vacuum followed by lyophilization.

The dried leachate weights were recorded and each sample was redissolved in water to a concentration of 5 mg/ml for LC-UV-MS injection in 5 μl volumes.

2.3. Optimization of leaching in ethanol

Quinoa seeds (1.6 g) were incubated in 8 ml aqueous ethanol at varying concentrations (0, 25, 33, 40, 50, 60, 70, 80 or 95%) in 15 ml tubes on a shaker at 200 rpm/min in the dark over a range of temperatures (25 – 80°C) and time points (1 – 24 h). Seeds were leached for 24 h at lower temperatures (25 and 37°C) and up to 8 h at higher temperatures (50 and 80°C). Using the optimal solvent, temperature and time conditions, 20 minutes sonication and solvent acidification (1% acetic acid) were also tested for their effects on 20HE leaching. Leachates were filtered through 0.45 μm syringe filters into 8 ml glass vials and dried by speed vacuum followed by lyophilization. The dried leachate weights were determined. Aliquots of each sample were redissolved in their corresponding solvent (0– 95% ethanol) at a concentration of 5 mg/ml and injected in the LC-UV-MS in 1–5 μl volumes.

The optimal leaching conditions were determined by three factors: (1) the amount of 20HE released from the seeds into solution (μg 20HE/g seed), (2) the 20HE concentration in final dried leachate (μg 20HE/mg), and (3) the yield, or the amount of leachate obtained per g of seed (mg/g seed).

2.4. Seed extraction

Quinoa seeds were pulverized in a coffee grinder (Krups, Inc.). The seed powder (1.6 g) was mixed with 8 ml 70% ethanol for 4 h at 25 or 80°C, centrifuged at 4,000 rpm for 10 min at 4°C, filtered, dried, and analyzed via LC-UV-MS. In order to compare extraction of macerated seed powder with the one-step process of leaching intact seeds, the seed powder was also extracted at 80°C three times consecutively, using 5 ml of fresh 70% ethanol per g seed powder each time.

2.5. Production and analysis of quinoa leachate (QL) for biological study

Quinoa seeds (1 kg) were rinsed in cold water for 5 min and leached in 5 l of solvent under the optimized conditions (70% ethanol, 80°C, 4 h). The leachate was filtered through a Whatman filter paper, dried and weighed. An aliquot of QL was resuspended in 30% acetonitrile at a concentration of 20 mg/ml, filtered, and injected in the LC-UV-MS in 3 μl volumes for analysis of the total phytoecdysteroid and total flavonoid glycoside content. A separate aliquot of QL (30 g) was used for proximate nutritional and amino acid profile analysis by Eurofins Nutrition Analysis (De Moines, IA) according to standard methods. The concentration of the total phytoecdsyteroids and flavonoid glycosides in QL was subtracted from the crude fat/oil content, which was determined via extraction with diethyl ether and petroleum ether. The carbohydrate content was calculated as 100% minus the sum of the other phytochemical and nutritional components (phytoecdysteroids, flavonoid glycosides, protein, fat/oil, moisture, ash). The remaining QL was used for biological study.

2.6. Isolation and identification of quercetin trisaccharide I (QT-I)

The major flavonoid glycoside present in QL (indicated as peak 3 in Fig. 4. and listed as quercetin trisaccharide I in Table 2) was isolated as follows: QL (14 g) was suspended in water containing 1.0% acetic acid and applied to a pre-conditioned Phenomonex® Strata C18-E (55 μm, 70 Å) solid phase extraction (SPE) cartridge. QL components were eluted into 3 fractions using 3 bed volumes each of water, 70% ethanol and 95% ethanol, successively. Each elution solvent contained 1.0% acetic acid to maintain the flavonoid glycoside stability. The 70% ethanol fraction, determined to contain all the QL flavonoid glycosides by LC-UV-MS analysis, was dried, weighed (1.89 g) and further fractionated using Bench Scale Fast Centrifugal Partition Chromatography (FCPC) Kromatons v. 1.0. with a stationary phase of 4:1 BuOH:EtOAc and a mobile phase of water. Thirteen fractions were collected. QT-I, determined by LC-UV-MS to be concentrated in fraction 6 (FCPC 40 – 65 min, 247 mg), was isolated using preparative reverse phase HPLC (Waters 616 four channel pump with semi-preparative pump heads operated on a Waters 600 Controller; Waters 490E Programmable Multiwavelength Detector set to monitor at 254 nm; Waters 717 Plus Autosampler; Phenomenex® semi-preparative Synergi Hydro C8 column 4μM, 250 × 20 mm; isocratic 15:85 acetonitrile:water containing 0.1% TFA over 60 min, flow rate 10 ml/min), yielding 10.0 mg QT-I (90% pure, t_R = 27 min). QT-I was re-purified by RP-HPLC (isocratic 14:86 acetonitrile:water containing 0.1% TFA over 75 min, 10 ml/min), yielding 3.0 mg (99% pure, t_R = 50 min). The isolated compound was identified from its mass spectra, 1D and 2D NMR spectra (^{1}H, ^{13}C, COSY, DEPT, HMQC and HMBC) recorded on a Bruker Avance 700 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA), using CD_3OD as the solvent, and by comparison with data reported in the literature (Zhu, Sheng, Li, Lavoie, Karwe, Rosen, & Ho, 2001b).

2.7. LC-UV-MS analysis

Standards of 20HE (99% pure, Bosche Scientific, New Brunswick, NJ) and makisterone A (98% pure, A.G. Scientific, Inc., #M1-1080) were dissolved in 70% ethanol at concentrations of 1, 0.1, 0.05 and 0.01 mg/ml and used for chemical identification and quantification by LC-UV-MS at 247 nm with 1 μl injection volumes. QT-I (99% pure, isolated as described above) was dissolved in 95% ethanol at the same concentrations and used as an external standard. Compounds for which no standards were available were putatively identified using their retention time, mass signal for the precursor ion ([M+H]$^+$, [M-H]$^-$) and fragment ions, fragmentation pattern and molecules reported in the literature. 20HE analogs (24-epi-makisterone A, 24(28)-dehydromakisterone A, and makisterone C) were calculated as 20HE equivalents (concentrations of individual molecules were estimated using a standard of 20HE). Concentrations of quercetin and kaempferol glycosides were calculated from the peak areas recorded at 247 nm to the calibration curve obtained with QT-I. Results of flavonoid concentrations were expressed as QT-I equivalents.

Analysis was performed using the Dionex® UltiMate 3000 RSLC ultra-high pressure liquid chromatography system, consisting of a workstation with Dionex's Chromeleon v. 6.8 software package, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS and photodiode array detector DAD-3000RS. After the photodiode array detector the eluent flow

was guided to a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass spectrometer with an electrospray ionization interface (ESI), operated in either positive (5 kV) or negative (-4.5 kV) ionization modes. The drying gas temperature was 280°C and nitrogen was the sheath gas. The mass detector was used in scanning mode from 65 to 1500 atomic mass units (amu). Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v. 6.41, SP2. Substances were separated on a Phenomenex® C8 reverse phase column, 150×2.0 mm, particle size 3 μm, pore size 100 Å. The mobile phase consists of 2 components: Solvent A (0.5% ACS grade acetic acid in ddH$_2$O, pH 3-3.5) and Solvent B (100% acetonitrile). The mobile phase flow was 0.2 ml/min, and a gradient mode was used for all analyses. The initial conditions of the gradient were 93% A and 7% B; the proportion reached 73% A and 27% B over 40 min; solvent B reached 100% in the next 5 min and was maintained for 2 min; the column was re-equilibrated to initial conditions for 13 min. The total run time was set to 60 min.

2.8. Animal study

An acute hypoglycemic study in diet-induced obese mice was performed as described in Cheng et al. (2012). This study was carried out in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Rutgers University (Protocol Number: 04-023). Briefly, five-week-old male C57Bl/6J mice were purchased from Jackson Labs, acclimated for 1 wk with *ad libitum* access to chow (Purina, No. 5014) and water, and maintained on a very high-fat diet (VHFD) containing 60% fat-derived calories (D12492; Research Diets) for an additional 15 wk to induce obesity, insulin resistance and hyperglycemia. The food intake and body weight were monitored weekly. Following a 4 h fasting period, the fasting blood glucose (FBG) was measured using an AlphaTRAK® handheld glucometer (Abbott Labs, Inc.), and mice were randomly divided into FBG-balanced experimental groups. QL was formulated in 70% Labrasol® (Gattefossé Corp., Paramus, NJ). The biological activity of QL was tested using an oral ingestion of 0.25 ml per 50 g body weight of vehicle (70% Labrasol®) or QL (250 and 500 mg/kg) (n=7). Metformin® (300 mg/kg, dissolved in water) was administered as a positive control. FBG was reassessed 4 h post-treatment, and the final % FBG for each mouse was calculated individually using the equation: $(\text{final FBG}/\text{initial FBG}) \times 100$. The mean final % FBG values for each treatment group were compared to the vehicle by 1-way ANOVA followed by Dunnett's post hoc test using Prism 6.0 (GraphPad Software, San Diego, CA). *P*<0.05 was considered significant.

3. Results

3.1. 20HE leaches from germinating seeds

20HE leached from quinoa seeds into water gradually over the course of 144 h, with greater leaching occurring at 37°C (294.0 μg/g seed) compared with at 25°C (156.5 μg/g seed) (Fig. 1). Seedlings germinated under all conditions. The greatest seedling growth occurred in the seeds incubated in the dark at 25°C after 96 hours. Light did not impact the amount of 20HE that was leached from the seeds compared with the seeds germinated in the dark.

3.2. Optimal leaching conditions

The optimal ethanol concentration for leaching 20HE from quinoa seeds at 25°C was 70% (Fig. 2A). Extracting the ground seeds in 70% ethanol at 25°C released 399 μg 20HE/g seed, whereas leaching intact seeds for 24 h released 317.8 μg 20HE/g seed (about 80% as efficient as extraction). Proportionally less seed material was solubilized into 70% ethanol during leaching compared to extraction. Therefore, the dried extracts and leachates had a similar 20HE content (10.4 and 10.7 μg 20HE/g seed, respectively) (Fig. 2B). The leachate yield, measured as the amount of dried leachate produced from seeds, was generally lower than that of the extract. For example, the leachate yield produced using 70% ethanol was 29.7 mg/g seed compared to 38.1 mg/g for the extract (Fig. 2C). The total amount of 20HE leached, concentration in the dry leachate and leachate yield all decreased dramatically in 95% ethanol.

The effect of temperature on the efficiency of 20HE leaching into 70% ethanol as compared to extraction at the same temperature was also studied. At temperatures above 25°C, more seed material, including 20HE, was leached into the solvent (Table 1). Compared with the total 20HE that was obtained from repeated extraction of seeds, leaching intact seeds in 70% ethanol at 80°C for 4 h released the maximum amount of 20HE available in the seeds (491 μg/g) (Table 1). Under these conditions, the leachate yield was 4.5% and the 20HE content of the dried leachate was 1.09%. Leaching was further optimized by time (1–8 h) and solvent to seed ratio (1–5 ml/g seed), demonstrating that 4 h of leaching and 5 ml solvent/g seed were the optimal conditions for leaching most of the 20HE available in seeds and obtaining the highest yield of QL (Supplementary Fig. 1). Sonication and solvent acidification did not affect the amount of 20HE leached from the seeds or the 20HE concentration in the dried leachate (data not shown).

3.3. Biochemical analysis of quinoa leachate (QL)

QL was prepared for phytochemical, nutritional, and biological study from 1 kg seed under optimal conditions, yielding 41.0 g QL. The phytochemical content was determined using LC-UV-MS (Table 2). Figure 3 shows the molecular structures and LC-UV chromatogram at 247 nm of the QL phytochemical components. Fifteen peaks corresponding with fifteen different molecules were putatively identified as listed in Table 2, including 4 quercetin glycosides, 1 kaempferol glycoside and 5 phytoecdysteroids (20HE, makisterone A, epi-makisterone A, 24(28)-dehydromakisterone A, and makisterone C). 20HE constituted 0.86% of QL, whereas the total phytoecdysteroid content was 1.00%. The major quercetin trisaccharide in QL (QT-I) was isolated as a yellow powder (99% pure,). Its MS showed a m/z 757 [M+H]$^+$ and m/z 755 [M-H]$^-$ as molecular ion peaks, in the positive and negative ionization modes, respectively. The compound was identified as quercetin 3-O-(2,6-di-α-L-rhamno-pyranosyl)-β-D-galactopyranoside according to its MS, MS/MS and NMR spectra, and in comparison with the spectra reported in the literature (Zhu, Sheng, Li, Lavoie, Karwe, Rosen, & Ho, 2001b). The total flavonoid glycoside content, determined as QT-I equivalents, was 2.59% of QL. Other components, determined by proximate nutritional analysis of QL, included carbohydrates (53.6%), protein (20.4%), oil (11.9%), ash (6.97%) and moisture (3.53%). About 0.37% of QL consisted of branched chain amino acids

(leucine, isoleucine, and valine) (Supplementary Table 1). LC-MS analysis of QL also revealed trace amounts of saponins (data not shown).

3.4 In vivo hypoglycemic effect

Acute oral administration of QL demonstrated a dose dependent decrease in FBG in diet-induced obese, hyperglycemic mice (C57BL/6J) with FBG levels above 200 mg/dL. Relative to the initial FBG levels of individual mice, 500 and 250 mg/kg QL decreased te FBG by an average of 36.2% and 18.5%, respectively. Metformin (300 mg/kg) decreased FBG by 26.4%. Metformin and the higher dose of QL (500 mg/kg) significantly decreased final % FBG compared with the control by 1-way ANOVA followed by Dunnett's post hoc test (Fig. 4).

4. Discussion

This work demonstrated for the first time that 20HE is leached or secreted from germinating quinoa seeds. At 37°C, approximately 60% of the total 20HE in intact quinoa seeds was leached into water. Phytoecdysteroids are structurally related to insect molting hormones, and have shown a range of effects on insects, including deterrence of leaf herbivory, modulation of insect taste receptors, the delay of larval development and lethality (Arnault & Slama, 1986; Blackford & Dinan, 1997; Marion-Poll, et al., 2002; Rharrabe, et al., 2010; Singh, et al., 1980). A range of soil insects that feed on seeds, seedlings and roots (wireworms, cutworms, thirps, grubs, flea beetles, billbugs and diabrotica beetles) are known predators of other Andean crops, such as maize and potatoes (O'Day, Becker, Keaster, Kabrick, & Steffey, 1998). It is tempting to speculate that phytoecdysteroid leaching plays a role in quinoa's defense from soil insects during seed germination and seedling production. The co-leaching of 20HE, minor phytoecdysteorids and other secondary metabolites (flavonoids, saponins) may provide quinoa seeds with complimentary, multitarget defense mechanisms against soil pathogens and herbivores.

The fast, efficient method described here for leaching and concentrating 20HE from quinoa seeds enhanced the 20HE content in QL 17.5-fold, compared with the concentration found in the seeds. Post-leached seeds retained their shape, form and colour. The seed by-products were sterilized by 4 h incubation in 70% ethanol, and still contained the majority of their macronutrient content following the leaching process (95.9% of initial seed weight). Post-leached seed material may still be used for human or animal nutrition. Post-leached quinoa seeds can be toasted and consumed directly as "popped quinoa", incorporated in health food bars and snacks as whole seeds, milled to produce quinoa flakes, or ground to make quinoa flour.

QL significantly decreased the blood glucose levels in a diet-induced obese, hyperglycemic mouse model. Acute studies in this model have been routinely used to demonstrate the *in vivo* anti-diabetic effects of several pharmacological agents and botanicals (Cheng, Kuhn, Poulev, Rojo, Lila, & Raskin, 2012; Grace, et al., 2009; Kellogg, et al., 2010; Ribnicky, et al., 2009; Rojo, et al., 2012; Roopchand, et al., 2012). The acute hypoglycemic effects of QL, combined with previous reports on the anti-diabetic and anti-obesity effects of chronic 20HE administration, support QL's potential to treat or prevent hyperglycemia and insulin

resistance associated with human metabolic syndrome (Foucault, Mathe, Lafont, Even, Dioh, Veillet, Tome, Huneau, Hermier, & Quignard-Boulange, 2011; Kizelsztein, et al., 2009).

Other components of QL, including flavonoids (quercetin and kaempferol glycosides), fatty acids and amino acids, may potentiate or synergize with 20HE's pathogen / herbivore-protective affect as well as its anti-diabetic activity. Flavonoids (2.59% of QL) have been widely reported to have antioxidant, anti-diabetic, anti-obesity, anti-hypertensive, anti-cancer and anti-inflammatory properties (Da-Silva, et al. 2007; Jeong, Kang, Choi, Kim, & Kim, 2012; Kelly, 2011; Saragusti, Ortega, Cabrera, Estrin, Marti, & Chiabrando, 2010). Quinoa oil (11.9% of QL) is largely composed of omega-3 and omega-6 fatty acids, which have been shown to improve insulin sensitivity and cardiovascular health (Vega-Galvez, et al., 2010). Meanwhile, amino acids like leucine (0.08% QL) may control blood sugar levels and weight gain in animals (Zhang, Guo, LeBlanc, Loh, Schwartz, & Yu, 2007), while promoting insulin secretion from pancreatic β cells (Yang, Chi, Burkhardt, Guan, & Wolf, 2010).

QL also contains trace amount of saponins. Saponins are sometimes considered toxic due to their hemolytic effect when in direct contact with the blood. However, saponins are known to have low toxicity following oral ingestion, are safe for topical exposure, and are even reported to have beneficial internal effects, including lowering cholesterol, enhancement of mucosal drug absorption, antimicrobial activity and anti-inflammatory effects (Kuljanabhagavad & Wink, 2009; Shi, Arunasalam, Yeung, Kakuda, Mittal, & Jiang, 2004). Most commercially available quinoa seeds are polished and washed to remove most saponins, and the seeds can be rinsed again before leaching to further reduce the saponin content in QL. A 2 g dose of QL would deliver a saponin content that is less than the intake associated with the consumption of 1 serving of quinoa, well within a safe range (Zevallos, Herencia, Chang, Donnelly, Ellis, & Ciclitira, 2014).

Our biochemical characterization corroborates previous reports that quinoa seeds contain relatively high levels of phytoecdysteroids. Zhu et al. (2001a) were the first to isolate and identify phytoecdysteroids from quinoa seeds, reporting 30 μg 20HE/g seed. However, a later study, which screened the 20HE content from various sources of quinoa seeds, found a range of 184 – 484 μg 20HE/g seed (Kumpun et al., 2011). 20HE constituted 61.9% and 88.6% of the total phytoecdysteroids isolated from quinoa seeds in these studies, respectively. In our study, AlterEco red quinoa seeds contained 491.3 μg 20HE/g seed, constituting 85.7% of total phytoecdysteroids.

As noted by Kumpun et al. (2011), 20HE levels in quinoa seeds can vary greatly depending on the source. However, the genetic and environmental factors affecting phytoecdysteroid content in quinoa seeds are currently unknown. Leaching in 70% ethanol may be a simple method to evaluate phytoecdysteroid content among differing quinoa seed sources.

5. Conclusion

We have demonstrated that quinoa seeds leach the majority of their stored phytoecdesteroids, along with several other biologically active compounds, such as

flavonoids, into the surrounding medium, possibly as a defense mechanism against soil insects. The leaching process was optimized to concentrate the anti-diabetic quinoa constituents. QL production leaves behind mostly intact quinoa seeds that still contain majority of their macronutrient reserves (protein, starch and oil), which can be recycled as food ingredients. Therefore, quinoa seed leaching may be a way to harness the clinically therapeutic benefits of quinoa phytochemicals while preserving the nutritional benefits of non-leached quinoa components. Our work contributes to the global initiative to promote the use of quinoa seeds in human health and sustainable development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ANOVA	analysis of variance
Ctl	control
FBG	fasting blood glucose
20HE	20-hydroxyecdysone
LC-UV-MS	liquid chromatography-ultraviolet-mass spectrometry
Met	metformin
QL	quinoa leachate
QT-I	quercetin trisaccharide I
SEM	standard error of the mean
t_R	retention time
VHFD	very high fat diet

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Highlights

- 20-Hydroxyecdysone (20HE) and flavonoids can be effectively leached from quinoa seeds into water or aqueous ethanol.

- Optimal solvent, temperature, and time conditions for leaching 20HE were established.

- The leaching procedure released essentially all 20HE available in the seeds (491 μg/ g seed).

- Quinoa leachate (QL) significantly lowered fasting blood glucose in obese, hyperglycemic mice.

- Quinoa leaching may be useful for the production of anti-diabetic botanical therapeutics.
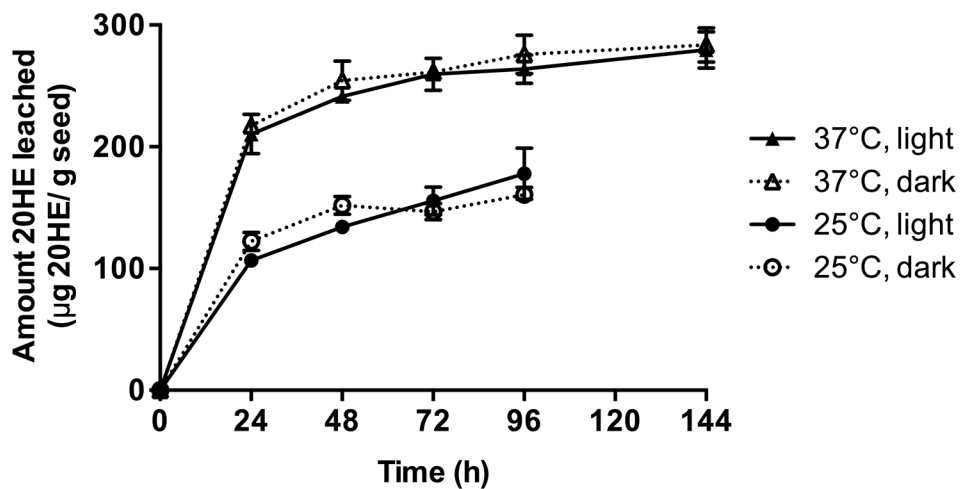
Figure 1. 20-Hydroxyecdysone (20HE) leaches from quinoa seeds into water cumulatively over time

AlterEco Red quinoa seeds were surface sterilized and immersed into sterile water for 24–144 h under light or dark conditions at 25 or 37°C. 20HE content was determined in dried leachates by LC-UV-MS. Data are the mean ± SEM (n=3).
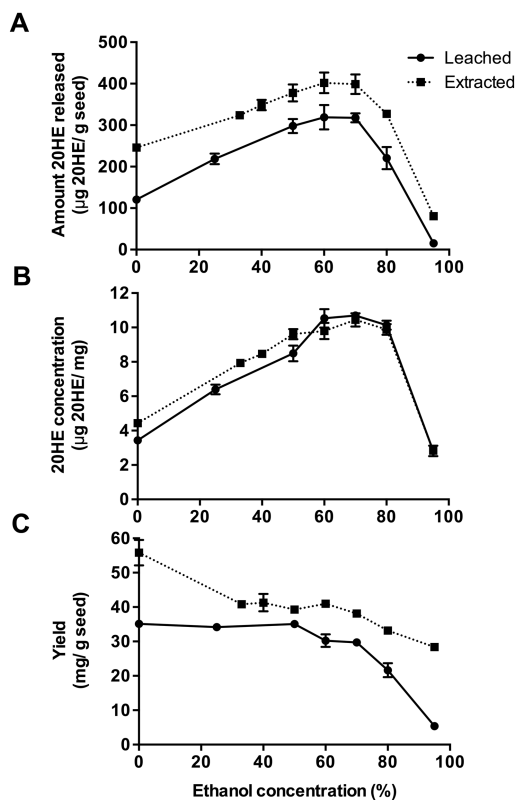
Figure 2. Leaching versus extraction of 20-hydroxyecdysone (20HE) from quinoa seeds into varying concentrations of ethanol

Intact quinoa seeds were incubated in ethanol (0–95%) at 25°C for 24 h to produce leachates, or macerated seed material was extracted in ethanol (0–95%) at 25°C for 4 h. The 20HE content was determined in dried leachates and extracts by LC-UV-MS. Three parameters were measured, shown from top to bottom: (A) amount 20HE released from seeds (μg 20HE/g seed), (B) 20HE concentration in the final dry leachate or extract (μg 20HE/mg), and (C) yield of dry leachate or extract (mg/g seed). Data are the mean ± SEM (n=3).
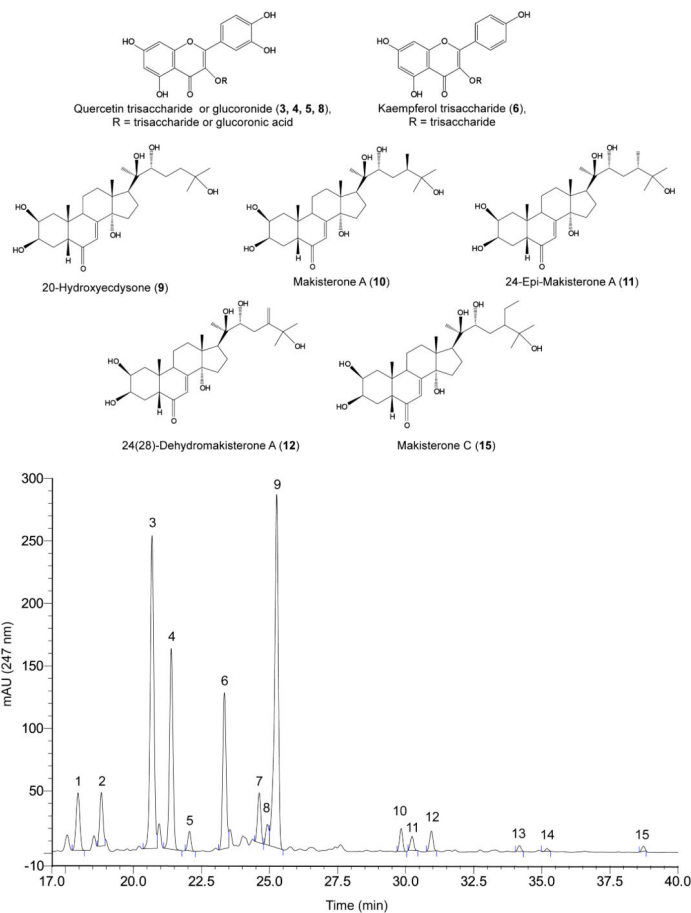
Figure 3. Molecular structures and LC-UV chromatogram at 247 nm of phytochemicals putatively identified in QL

QL was dissolved in 30% acetonitrile (20 mg/ml) and 3 μl was injected for chemical characterization alongside standards of 20HE, makisterone A and quercetin trisaccharide I via LC-UV using a C8 column with a gradient of 7 to 27% acetonitrile over 40 min. Compounds were putatively identified by UV retention time, MS *m/z* and fragmentation pattern, and based on quinoa compounds reported in the literature. Numbers indicate peaks corresponding with the compounds listed in Table 2.
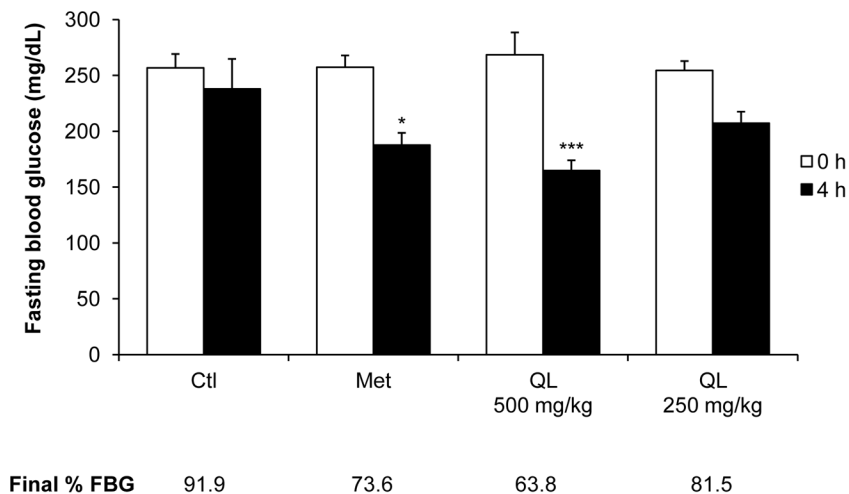
Figure 4. Quinoa leachate (QL) lowers fasting blood glucose (FBG) in obese, hyperglycemic C57BL/6J mice

QL was dissolved in 70% Labrasol (vehicle). FBG was determined via tail-nick before and 4 h after administration of vehicle (Ctl), 300 mg/kg Metformin® (Met, positive control), or QL (250 and 500 mg/kg). Data are the mean ± SEM (n=7). Values reported below the x-axis are the results of mean final % FBG for each group. Final % FBG was calculated for individual mice as post-treatment FBG/ pre-treatment FBG x 100. *$P < 0.05$ and ***$P < 0.001$ (1-way ANOVA followed by Dunnett's post hoc test compared to control).

Table 1

Leaching versus extraction of 20-hydroxyecdysone (20HE) from quinoa seeds at various temperatures.

	Leached				Extracted		
Temperature (°C)	25	37	50	80	25	80	80
Time (h)	24	24	4	4	4	4	4[a]
Amount 20HE released (μg 20HE/g seed)	318 (±11.0)	327 (±35.9)	368 (±4.8)	491 (±1.0)	399 (±23.6)	423 (±1.6)	449 (±18.8)
20HE concentration (μg 20HE/mg)	10.7 (±0.2)	9.2 (±0.9)	11.4 (±0.2)	10.9 (±0.1)	10.4 (±0.4)	9.3 (±0.4)	6.2 (±0.2)
Yield (mg/g seed)	29.7 (±0.7)	35.6 (±0.6)	32.3 (±0.3)	45.0 (±0.5)	38.1 (±0.9)	45.8 (±1.8)	72.4 (±1.0)

Intact quinoa seeds were incubated in 70% ethanol (5 ml/g seed) at increasing temperatures (25, 37, 50, or 80°C) for 4 or 24 h. For comparison, macerated seed powder was extracted in 70% ethanol (5 ml/g seed powder) at 25°C (single extraction) or 80°C (single extraction, or the same seed powder was extracted 3 times consecutively with fresh solvent). Leachates and extracts were dried and analyzed for 20HE content by LC-UV-MS. Data are the mean±SEM (n=3).

[a] Macerated seed material was extracted 3 times consecutively.

Table 2

Retention time, putative chemical identification, *m/z* and concentration of phytochemicals in quinoa leachate (QL).

Peak No.[b]	t$_R$ (min)	Compound	[M+H]+ (*m/z*)	[M-H]− (*m/z*)	Fragment (*m/z*)	Conc. (mg/g)
1	17.97	Triterpenoid derivative I		664[c]		-
2	18.82	Triterpenoid derivative II		664[c]		-
3	20.68	Quercetin trisaccharide I[d]	757	755	611, 303	11.68
4	21.39	Quercetin trisaccharide II	743	741	611, 465, 303	7.21
5	22.06	Quercetin trisaccharide III	743	741	611, 303	0.69
6	23.34	Kaempferol trisaccharide	741	739	595, 449, 287	5.59
7	24.62	Triterpenoid derivative III		609[c]		-
8	24.92	Quercetin glucoronide	479		303	0.72
9	25.26	20-Hydroxyecdysone	481	539[c]	463	8.60
10	29.83	Makisterone A	495		477, 459, 441	0.46
11	30.23	24-Epi-makisterone A	495		477, 459, 441	0.30
12	30.94	24(28)-Dehydromakisterone A	493		475, 457, 439	0.45
13	34.18	Ecdysteroid	465	464	447, 429	0.12
14	35.19	Steroid	427			-
15	38.73	Makisterone C	509		491	0.10
		Total phytoecdysteroids				10.03
		Total flavonoid glycosides				25.89

tR= Retention time

[b] Peak number, refer to Fig. 3

[c] The reported [M-H]− represents an acetic acid adduct [M-H+C2H4O2]−.

[d] Quercetin trisaccharide I (QT-I) was identified as quercetin 3-*O*-(2,6-di-α-L-rhamno-pyranosyl)-β-D-galactopyranoside.