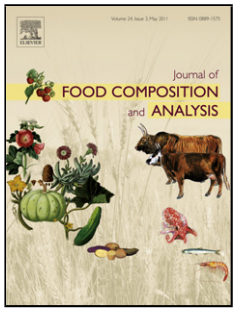

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Physicochemical characterization of jicaro seeds (*Crescentia alata H.B.K.*): a novel protein and oleaginous seed

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Highlights

- Jicaro seed is a novel biodverse food crop rich in high quality oil and protein similar to soybean
- Jicaro fatty acid and amino acid profiles are of nutritional interest
- Absence of anti-nutritional factors (anti-trypsin and α-galactosides)
- Jicaro is a high quality food source resistant to nutrient-poor soils and intense drought

ABSTRACT

Jicaro (*Crescentia alata*) is a tropical tree native to Mesoamerica well adapted to severe drought conditions. The seeds of the fruits were analyzed for protein, fatty acid, dietary fiber, phytate, polyphenol, tannin, tocopherol, sugar, mineral, amino acid and trypsin inhibitor contents. The jicaro cotyledons contained 43.6±1.15 g protein/100 g and 38.0±0.20 g fat/100 g (d.w.), which is comparable to most protein-rich and oleaginous seeds. Among the lipids, 71.2% were unsaturated fatty acids, particularly oleic acid, and essential amino acids represented 16.0±0.9% (d.w.) of the protein fraction, which is similar to soybean amino acid contents. A proteomic analysis and SDS-PAGE electrophoresis revealed that the proteins are mostly of low molecular weight (~10 kDa), and the storage protein 2S albumin dominated. Jicaro seed trypsin inhibitory activity was low (0.1 trypsin inhibitor units TIU/mg), which enhances the digestibility of its proteins. The jicaro seed cotyledon represents an autochthonous and high-quality food source.

Keywords

Food analysis, Food composition, *Crescentia alata*, Nutritional quality, Jicaro seeds, Biodiverse food , , oleaginous seed, , Oil profile, Proteomic..

1. Introduction

Jicaro (*Crescentia alata*), which belongs to the family Bignoniaceae, is an ancient tree of American tropics (Vázquez-Yanes et al., 1999). Jicaro fruit has a calabash shape, and it was widely used during pre-Columbian era not only as a staple food but also as food utensils and containers. Jicaro fruit has a lignified pericarp that contains a sweet pulp surrounding many seeds. Jicaro fruit seeds, which represent 5% of the whole fruit, are dark brown, flat and heart shaped with a pleasant characteristic odor. Little research concerning jicaro exists, and few studies have attempted to characterize the composition of jicaro seed. Several decades ago, oil and fatty acid composition of jicaro seed was partially determined with respect to its main fatty acids (61.8% oleic, 15.0% linoleic) (Lewy, 1960). A more recent work performed on Guatemalan fruits showed that the seeds contained 38% fat and 26% protein (Figueroa and Bressani, 2000). These preliminary data were nonetheless of very high importance, as even in silvopastoral systems, with an average of 350 trees per hectare, each tree produces 750 fruits per year corresponding to 52 kg of fresh seeds (Carballo et al., 2005), which represents a high potential for commercial production. Additionally, the tree is well adapted to Pacific region of Central America, where severe droughts can occur, and thanks to deep root system, jicaro can absorb nutrients and water from savanna soils (Bucheli et al., 2013). Jicaro is one of the only trees to remain green year-round in the dry regions of Central America.

Jicaro seed was an important staple food in some pre-Columbian communities (Staller, 2010), and it is still widely consumed in Mesoamerica, especially in El Salvador and Nicaragua, as a very popular beverage called “horchata”. Nonetheless, because of real value of this seed remains unknown, consumption is decreasing and preservation of jicaro trees in silvopastoral systems must be strengthened. In the context of adaptation to climate change, a renewed interest in sustainable and biodiverse food sources with high nutritional and functional potential, has led to the rise of new market opportunities. For this reason, the

objective of the present study was to describe the physicochemical characteristics of jicaro seeds and their main nutritional properties.

2. Materials and methods

2.1. Raw materials

Jicaro seeds (*Crescentia alata*) were obtained from the village Caserio Los Zarzarles (latitude: 12.66°N, longitude: -86.44°W, altitude: 115.7 m) in the municipality of León during two harvesting season, november 2013 and 2014. The seeds were prepared following a traditional process. Mature fruits were halved, and the seed-containing pulp was placed in polystyrene bags to ferment for three days. The seeds were then separated and washed before being sun dried. A total of 100 kg of dried seeds were collected from different jicaro fields, gathered and mixed at the Universidad Nacional Autónoma de Nicaragua (UNAN) of the city of Leon in the Pacific coast. . Foreign material was removed. The seeds were washed again and sun dried to obtain a unique homogenized batch, representative of the overall production. All analyses were performed on samples of this representative batch.

Cotyledons and seed coats were separated manually from jicaro seeds. Each seed part was ground separately into a powder by using a PREP'LINE knife blender (SEB, Ecully, France) before analysis. Commercial yellow soybean (Markal, Saint-Marcel-lès-Valence, France) was bought in a supermarket in Montpellier (France) and was used as control.

2.2. Chemical analysis

Jicaro seed, cotyledon and seed coat samples were analyzed in triplicate. Dry matter content of 5 g sample was determined according to PR NF ISO 6496 standard using differential weighing after oven drying at 103°C to reach a constant weight.

Micro-Kjeldahl method was used according to the Official Method 950.48 (AOAC, 1995) to determine total protein content (Nx6.25) with an automatic Foss analytical AB Kjeltec^{TM} 8400 apparatus (Foss, Höganäs, Sweden). Each sample was previously mineralized with concentrated sulfuric acid and a mixture of 0.15 g of $CuSO_4 \bullet 5H_2O$, 5.0 g K_2SO_4, and 0.15 g titanium dioxide (Thompson Capper Ltd., Hardwick, UK) (Venkatachalam and Sathe, 2006).

Fat content was determined by using an ASE™ 350 accelerated solvent extractor (DIONEX Corp., Sunnyvale, CA, USA). A 1 g sample was placed in the stainless steel extraction cell in the presence of 0.5 g sand. The extraction solvent was pure petroleum ether at 60°C and 100 bar. Flush was set to 100%, number of cycles to 5 with a static time of 7 min, and purge duration was 90 sec. The extraction solvent containing lipids was collected in a 50 mL flask that had been previously dried and weighed. Flasks were weighed again after total evaporation of the solvent. Lipid content was expressed as g fat per 100 g sample (dry weight basis).

2.3. Soluble sugars

Sugar analysis was performed on the lipid-free meal remaining in the extraction cell. A 80% ethanol solution (v/v) was used as the solvent at 60°C and 100 bar. Extracts of recovered sugars were diluted 25-fold with deionized water and filtered to 0.45 µm before injection of 10 µL into the chromatographe. Soluble sugars were separated using high performance ionic chromatography (HPIC) with a DX600 system equipped with a Carbopac MA-1 column (250

x 4 mm), a Carbopac MA-1 guard column (25 x 4 mm) and a Dionex ED50 pulsed amperometric detector (PAD) (DIONEX Corp., Sunnyvale, CA, USA). Chromatographic conditions were described by Valente et al. (2013).

The determination of α-galactosides (raffinose, stachyose and verbascose) was performed according to a procedure described by Muzquiz et al. (1999). A sample containing phenyl α-D-glucoside (100 μg) as an internal standard and 48% aqueous ethanol was added. Extraction was performed using a sonication for 60 min, followed by centrifugation at 700×*g* for 10 min. Combined supernatants were heated at 85°C under reflux for 30 min, cooled and centrifuged at 700×*g* for 5 min. The supernatant was evaporated to dryness. The residue was dissolved in water, and an aliquot was transferred into a glass-stoppered test tube. Acetonitrile (1.0 mL) was then added with shaking, and the mixture was stored overnight at 4°C. The sample was injected into an HPLC DX600 system (DIONEX Corp., Sunnyvale, CA, USA).

2.4. Fiber content

Neutral detergent fiber was prepared from 1 g sample according to the method of Van Soest et al.,(1973) ,which sequentially yields neutral detergent fibers (NDF), acid detergent fibers (ADF) and acid detergent lignins (ADL). For NDF, a neutral detergent solution (sodium lauryl sulfate, USP-grade and ethylenediaminetetraacetic acid, EDTA; pH 7) at boiling temperatures with a 0.2 mL of heat-stable α-amylase Termamyl® (Laboratoires HUMEAU, La Chapelle-sur-Erdre, France) was used to dissolve the easily digested pectins and cell contents (proteins, starch sugars, and lipids), leaving a fibrous residue NDF (cellulose, hemicellulose and lignin). For ADF, 100 mL an acid detergent solution (20 g cetyl trimethylammonium bromide and 0.5 M H_2SO_4) were used to dissolve hemicellulose and minerals. Finally, for ADL, a 3 h digestion was performed with 72% H_2SO_4 in a crucible.

Waste mineralization was performed at 550 °C for 4 h. The results are reported on a dry matter basis, as cellulose (ADF-ADL), hemicellulose (NDF-ADF) and lignin (ADL).

2.5. Mineral composition

Mineralization (500 °C) of the sample in an ash furnace (Thermo Scientific™ Thermolyne™ 6000 series 408, Waltham, Massachusetts, USA) was performed prior to the analysis of P, K, Na, Ca, and Mg and similarly for trace elements (Fe, Mn, Cu and Zn) until the ashes were cleared. Ash was then digested with hot concentrate hydrochloric acid until the destruction of organic matter, as described by Pinta, (1973) methodology. Mineral contents were performed by inductively coupled plasma atomic emission spectroscopy ICP - AES) (Varian Vista-Pro, Palo Alto, CA, USA) and quantified against standard solutions of know concentrations. Total mineral content was defined as the sum of all the minerals analyzed.

2.6. Phytate determination

Phytate content was measured as described by Sekiguchi et al., (2000). Jicaro seeds (0.1 g) were placed in 2 mL 0.5 M HCl overnight at room temperature with constant stirring. After centrifugation (10 min, 10,000×*g*, 10 °C), the supernatant was recovered and then diluted in 0.2 M borax buffer (pH 8.0). Phytate content (inositol hexakisphosphate, IP6) was measured using HPIC with a DX600 system, equipped with an ATC-1 trap column, an AG11 guard column and an AS11 column, detection was performed with a conductivity cell connected to an ED50 detector after removing anions on an ASRS 300 (DIONEX Corp., Sunnyvale, CA, USA). The injected volume was 10 µL. Phytate elution was performed using a basic gradient of 200 mM NaOH. The gradient was linear from 30 to 80 mM NaOH for 8 min, before returning to and holding initial conditions for 8 min to re-stabilize the system. Data were analyzed with Chromeleon 6.0 software (DIONEX Corp., Sunnyvale, CA, USA). Calibration was done by using an external IP6 standard.

2.7. Total phenolic content

Total phenolic content was evaluated at 760 nm with the Folin-Ciocalteu reagent as described by Singleton, Orthofer & Lamuela-Raventos (1999). The lipid fraction was removed with hexane, and polyphenol extraction was performed on a 0.5 g sample added with 10 mL of acetone/water/formic acid (70/29/1, v/v/v). The results, in triplicate, are expressed as mg of gallic acid equivalents/100 g on a dry weight basis (mg GAE/100 g).

2.8. Tannin determination

Tannins were determined according to the ISO AFNOR NF V03-75 standard. They were extracted from a 1 g sample with dimethylformamide. After centrifugation (10 min, 10,000×*g*) and addition of iron (III) ammonium citrate, the absorbance of the supernatant was measured using spectrophotometry at 525 nm. The results are expressed as mg of tannic acid equivalents/100 g on a dry weight basis.

2.9. Tocopherol analysis

Tocopherols were extracted by using a method described by Taylor et al., (1976)and modified by (Deiana et al., 2002). A 0.5 g oil sample was added with 2 mL ethanol/pyrogallol (1%) and heated in a water bath at 70 °C for 2 min. Saponification was performed with 1 mL 12 N KOH for 30 min at 70 °C. Samples were extracted twice with n-hexane. Hexane phase was evaporated to dryness, suspended in absolute ethanol and filtered through a 0.45 µm micropore nylon membrane before injection. Tocopherols were analyzed according to the modified method described by Rossetti et al. (2010) using HPLC with a Dionex Ultimate 3000 system (DIONEX Corp., Sunnyvale, CA, USA) equipped with an Uptispher C18-HDO column 250x4.6 mm, 5 µm (Interchim, Montluçon, France). The injection volume was 20 µl.

The elution was performed isocratically with ethanol/methanol 60/40 (v/v) at a flow rate of 0.8 mL/min at 25 °C. Detection was performed with a fluorescence detector at 296 nm and 330 nm.

2.10. Fatty acid composition

Fatty acid composition was determined as described by Piombo et al. (2006). Oil samples (10 mg) were added with 3 mL sodium methylate solution with phenolphthalein in round bottom flasks. Hydrochloric methanol (3 mL) was added until phenolphthalein discoloration was observed. Hexane (8 mL) and water (10 mL) were added. The organic phase was recovered, dried over anhydrous sodium sulfate, and filtered for subsequent GC analysis using an Agilent 6890 chromatograph (Agilent, Santa Clara, CA, USA) equipped with an Innowax capillary column 30 m x 0.32 mm x 0.25 µm (Agilent, Santa Clara, CA, USA). Fatty acid methyl esters (FAME) were directly injected into the GC (He 1 mL/min, split 1:80). Injector and FID detector temperatures were 250 °C and 275°C respectively. The oven was heated from 185 to 225 °C at 5°C/min and held at 225 °C for 20 min. Fatty acids were identified by comparing their retention times with the FAME standards (Sigma-Aldrich, St. Louis, Missouri, USA).

2.11. Amino acid composition

Free amino acids were analyzed following the method used by Moore et al., (1958) Total amino acid analysis was performed using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). This system uses ion exchange chromatography with post-column ninhydrin derivatization and photometric detection with dual-wavelength measurements. The amino acid separation along the cationic column was obtained with a

succession of four sodium citrate buffers of increasing pH (2.6–8.6) and ionic strength (0.2–0.5 M) and with an increasing temperature gradient (52–95 °C). Amino acids were derivatized with the ninhydrin reagent (135 °C) and detected simultaneously at 570 nm and 440 nm. The entire process lasted 90 min per sample, including the resin regeneration phase. Quantification was performed by comparing peaks areas with a complete standard including 26 amino acids acidic, neutral and basic amino acids (Sigma, St. Louis, Missouri, USA). Norleucine (250 nmol mL^{-1} in sodium citrate buffer, 0.2 M, pH 2.2) was also used as an internal standard.

2.12. Proteomic profiling

Jicaro seeds (10 g) were ground under liquid N_2 using a mortar and pestle. Frozen pulverized plant material (300 mg) was divided in two aliquots and extracted with 500 µL 0.1 M Tris-HCl buffer (pH 6.8). After centrifugation (10,000×*g* for 5 min, 4 °C), the supernatants of both aliquots were combined and used for protein fractionation. Total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Berkeley, CA, USA) with bovine serum albumin (BSA) standards.

The crude extract (1 mg in 200 µL) was fractionated using reverse-phase high-performance liquid chromatography on a C_{18} column 4.6 x 250 mm, 5 µm particle diameter (Teknokroma Barcelona, Spain) with an Agilent 1200 chromatograph monitored at 215 nm (Agilent, Santa Clara, CA, USA). The flow rate was 1 mL/min, and the elution was performed by applying a gradient from 0.1% trifluoroacetic acid (TFA) in water (solution A) to acetonitrile containing 0.1% TFA (solution B) as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described by Lomonte et al.,(2014). The fractions were collected manually and evaporated by vacuum centrifugation (Eppendorf Vacufuge, Germany) at 45 °C. Once dried, the samples were reconstituted in water and mixed with reducing buffer for further separation by sodium

dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% pre-cast gradient gels (Bio-Rad, Berkeley, CA, USA). Protein bands were stained with Coomassie G-250 and digitally recorded in a Chemidoc® imaging device (Bio-Rad). Then, protein bands were excised from the gels, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with sequencing-grade bovine trypsin for 16 h in an automated digester (Progest, Digilab). Resulting peptides were mixed with an equal volume of a saturated solution of α-cyano-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA, and 1 μL of each peptide mixture was spotted onto an Opti-TOF 384-well plate, dried, and analyzed in positive-reflector mode using MALDI-TOF-TOF mass spectrometry model 4800 Proteomics Analyzer Plus instrument (Applied Biosystems, Foster City, CA, USA.). TOF spectra were acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected, and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix® standards (ABSciex, Framingham, Massachusetts, USA) spotted onto the same plate. For protein identification, resulting spectra were searched against UniProt/SwissProt databases (general and Viridiplantae) using ProteinPilot® v.4 and Paragon® algorithm (ABSciex, Framingham, Massachusetts, USA) (≥ 95% confidence), and spectra were submitted to MASCOT public server (Matrix Science). Peptide sequence spectra with lower confidence scores were manually interpreted and searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein similarity and family assignment. A few tryptic digest samples were analyzed by direct infusion in nano-ESI-MS/MS using a QTrap3200 instrument (Applied Biosystems, Foster City, CA, USA) operated in positive mode. Doubly or triply charged peptide ions were fragmented by collision-induced dissociation, and the resulting MS/MS spectra were interpreted by manual *de novo* sequencing.

2.13. Trypsin inhibitor

Trypsin inhibitor activity was assessed according to the method described by Stauffer (1990). The determination was based on the decrease in the hydrolysis rate of substrates (added bovine trypsin) caused by the inhibitor. The method involves extraction at pH 9 of the inhibitors which were added with bovine trypsin. The activity of the remaining trypsin is measured with N-α-benzoyl-D,L-arginine p-nitroanilide hydrochloride (BAPNA) under standard conditions. The amount of p-nitroaniline formed during a 10 min incubation is measured spectrophotometrically, and absorbance values in its presence and absence are used to calculate the number of trypsin inhibitor units (TIU) per milligram of sample.

3. Results and discussion

3.1. Seed structure

As depicted in **Figure 1**, the structure of jicaro seed consists of a brown seed coat, two white cotyledons stuck together and surrounded by a thin translucent envelope (cuticle). The seeds were small, thin, brown coated, and had an average weight of 53.3 ± 0.04 mg. The cotyledons represented 68% of the total seed weight. The seed coat, which accounts for approximately 32% of the seed weight, provides an effective protective layer.

3.2. Main chemical composition

The main chemical composition of jicaro seed is shown in **Table 1.** The main characteristic of the whole seed was its high protein and lipid contents, comparable to values reported previously by Figueroa and Bressani (2000). Protein and lipid contents in jicaro cotyledon reached nearly 44 and 38% (d.w.) respectively. Similar protein contents were found in soybean cotyledon (**Table 1)** but lower lipid contents were reported by El Tinay et al., (1989). Therefore jicaro seed could be defined as an oilseed thanks to its high content of lipids

and proteins comparable to sunflower, rapeseed, cotton and peanut seeds (Rodrigues et al., 2012).

Sugar composition of jicaro seeds is shown in **Table 1**. Sucrose was the major sugar component followed by fructose (5.15 and 0.42 g/100 g d.w. respectively in the seed, and 5.5 and 0.50 g/100 g d.w. respectively in the cotyledon). Sugar composition in soybean was determined in eight varieties using NMR proton nuclear magnetic resonance (^{1}H-NMR spectroscopy). The results show that sugar content depends on the variety and they showed low concentrations with respect to jicaro, except for glucose that is higher in the range 0.06 to 0.11% (Song et al., 2013). Another study on almond cultivars from Mallorca and California also confirmed that sucrose is the major sugar component in kernels (Egea et al., 2009; Yada et al., 2013). Sucrose in peanuts accounted for the major fraction with a mean value of 4.6% (Bishi et al., 2013) Oligosaccharides (e.g. stachyose, raffinose and verbascose), which are considered indigestible (Wongputtisin et al., 2015), were not found in jicaro seed (less than 0.1 g/100 g d.w.). At the opposite, soybean contained high amounts of raffinose and stachyose (6.62 g and 3.43 g/100 g d.w. respectively), and also some verbascose (0.13 g/100 g d.w.).

Neutral detergent fiber (NDF) was the main constituent of seed coat with more than 70 g/100 g d.w. compared to only 13.3 g/100 g d.w. in cotyledon. Cellulose-like compounds were dominant in the seed coat and represented about half of fibers (34.2 g/100 g d.w.), followed by hemicellulose and lignin (19.4 and 17.6 g/100 g d.w. respectively). In the cotyledon, hemicellulose, cellulose and lignin accounted for 8.3 and 3.3 and 2.2 g/100 g d.w. respectively. These values are comparable to those found in soybean cotyledons (**Table 1**).

The total mineral content of jicaro cotyledon (3.3 g/100 g d.w.) was comparable to that of soybean (3.5 g/100 g d.w.) and similar to the contents reported for melon, pumpkin and gourd seeds (Adeyemi and Adediran, 1994). Jicaro cotyledon exhibited a significant concentration of phosphorus (1.24 g/100 g d.w.), potassium (0.71 g/100 g d.w.) and

magnesium (0.52 g/100 g d.w.). Higher amounts of potassium were found in soybean cotyledon (1.97 g/100 g d.w.).

Phytic acid (PA) content was above 1000 mg/100 g of myoinositol hexakisphosphate (IP6). PA is a natural plant antioxidant constituting 1 to 5% of most cereals, nuts and oilseeds (Graf, 1990) and has the ability to chelate mono and bivalent metal ions, especially zinc, calcium, and iron. PA is the principal storage form of phosphorus in plant seeds. When iron and zinc bind to PA, they form insoluble precipitates and are far less absorbable in intestines. This process may therefore contribute to iron and zinc deficiencies in people whose diets rely on these foods for their mineral intake (Ruel and Bouis, 1998); (Hunt, 2003). However, health functionality of PA is still controversial, since some studies indicated a decrease of osteoporosis risk (López-González et al., 2013) and colon cancer with PA consumption via whole grains (Slavin, 2003).

Table 1 listed the main antioxidant compounds found in jicaro seed. Phenolic and tannin contents were low in all parts of jicaro seed. Higher values were found in soybean seed (Malenčić et al., 2012; Pedrosa et al., 2012). Tocopherols (γ-tocopherols δ/β-tocopherols and α-tocopherols) were quantified. γ-tocopherols is the most predominant form in the jicaro seed and cotyledons (11.3 to 8.82 mg/kg of dry weight, respectively) but relatively low concentrations if compared to soybean (15.92 mg/kg). Among tocopherols, α-Tocopherols is present in a low concentration (0.45 to 5.45 mg/kg) in jicaro cotyledons and soybean respectively, because the tocopherols' composition and other antioxidant compounds (polyphenol and tannins) their contents depend most often on the species, the genetic variability and the effect of environmental stresses on the growth and development of the seed (Britz and Kremer, 2002; Kumar et al., 2009; Tuberoso et al., 2007)

3.3. Fatty acid composition

Fatty acid profile of jicaro seed is shown in **Table 2**. Monounsaturated oleic acid (56.5% of lipid fraction) was the predominant fatty acid in jicaro cotyledon. Similarly high contents were observed in almond oil (62–80%) (Yada et al. 2011). The second most abundant fatty acid was polyunsaturated linoleic acid at almost 20% of the lipid fraction. In soybean, the reverse was observed with 22.2 and 52.5% of oleic and linoleic acids respectively. Saturated fatty acids such as palmitic acid and stearic acid represented 15 and 5% of jicaro lipid fraction, respectively. The content of polyunsaturated α-linoleic acid was only 2.5% in the jicaro lipid fraction. The other fatty acids were only present in trace amounts. Fatty acid profile of jicaro seeds from Nicaragua was similar to that obtained with jicaro seeds from Guatemala (Espitia-Baena et al., 2011). Jicaro seed was found to be rich in monounsaturated and polyunsaturated fatty acids, which accounted for more than 75% of the lipid content. Therefore, jicaro seed oil is potentially of high nutritional quality.

3.4. Amino acid and protein compositions

The major amino acid found in jicaro cotyledons (**Table 3**)was the essential amino acid leucine with a concentration up to 2.58 g/100 g d.w (7.4% of total amino acids). This value is similar in soybean cotyledon (3.23 g/100 g d.w.). The percent composition of the other essential amino acids in jicaro cotyledons, such as phenylalanine, valine, histidine, threonine, and methionine, were similar to those obtained with soybean cotyledon. Only lysine appeared at a much higher level in soybean cotyledon than in jicaro. The composition of non-essential amino acids was also very similar to that of soybean although essential to non-essential amino acid ratio was slightly lower (0.44%) in jicaro cotyledon.

3.5. Proteomic analysis of jicaro cotyledon

The crude protein extract of jicaro cotyledon which was extracted with no detergent and reducing agent, was resolved into 38 peaks via RP-HPLC (**Fig. 1A**). The peaks that eluted first (with a retention time < 22 min) did not correspond to proteins, as shown by SDS-PAGE analysis (**Fig. 1B**), and are probably metabolites or peptides, which are expected to elute early in the chromatogram. Protein peaks were further separated by SDS-PAGE under reducing conditions, resolving into more than 45 distinct protein bands that ranged from 10 to 110 kDa (**Fig. 1B**). The higher diameter of the spots at lower molecular weights (MW) observed in SDS-PAGE gel indicates a major proportion of low MW proteins in jicaro cotyledon (~10 kDa). Soybean storage proteins which are composed of two major components, β-conglycinin (7S globulin) and glycinin (11S globulin) have higher MWs (150–200 and 300–380 kDa respectively). After extraction in the presence of a detergent and a reducing agent, SDS-PAGE showed that β-Conglycinin was composed of three subunits with MWs of 80 kDa, 75 kDa and 50 kDa respectively and glycinin was composed of six subunits, each made up of an acidic polypeptide chain with a MW of 34-40 kDa linked by a disulfide bond to a basic polypeptide chain with a MW of 15-20 kDa (Medic et al., 2014; Mujoo et al., 2003; Yaklich, 2001)

A MALDI-TOF-TOF analysis of the trypsin-digested bands provided some peptide sequences that matched, with confidence levels of over 95%, those of known plant proteins reported in international databases (**Table 4**). The similarity of jicaro protein sequences to those from other plant genera facilitated their assignment to protein families associated with particular biological activities.

The sequence of the protein band that dominated in jicaro cotyledon and had an average MW of 11 kDa (peak 24, **Table 4**) matched the 2S albumin sequence. As storage

proteins, the 2S albumins are water-soluble and highly abundant proteins used by the plant as a source of nutrients, mainly nitrogen and sulfur, during germination (Agizzio et al., 2006).

As shown in **Table 4**, the other protein families identified by sequence similarity are specific to embryogenesis, but heat shock proteins were also detected, which may explain the exceptional resistance of jicaro to thermal and other environmental stresses such as drought (Coelho and Benedito, 2008); (Wang et al., 2004).

Proteomic analysis also revealed enzymes such as glucose and ribitol dehydrogenase homolog-1 and aldose reductase. These enzymes were found in cotyledon seeds and are involved in carbon metabolism and acquisition of desiccation tolerance, which are characteristics of seed development (Gallardo et al., 2003). It is worth noting that none of the sequences matched the trypsin or chymotrypsin-inhibitor sequences reported in the international database. This observation was consistent with jicaro seed antitrypsin activity which was found to be very low (< 0.1 TIU/mg), in contrast with soybean (43.5 TIU/mg). This low value of antitrypsin activity in jicaro seeds may enhance the digestibility of protein and the bioavailability of amino acids (Sarwar et al., 2012).

For the different protein sequences reported in **Table 4**, it was not possible to find matches with already reported sequences in international databases. Jicaro is a non-model organism for which there is no information currently available in these databases. This is the first time that a proteomic profiling of jicaro seeds is reported.

4. Conclusions

The characterization of jicaro seed (*Crescentia alata*) composition was completed, and these results highlighted the high potential of this novel, biodiverse food source due to its high oil and protein content. Another nutritional interest is its fatty acid and amino acid profiles but also the absence of anti-nutritional factors (anti-trypsin and α-galactosides). Nutritional characteristics of jicaro seeds were found very similar to those of soybean and other valuable

oils seed. Therefore jicaro might be considered as a substitute for these imported seeds promoting valorisation of local resources in these regions of Central America. Jicaro trees which grow in nutrient-poor savanna soils and extreme climates, could provide a high-quality food source to local population. Food industries typically ignore local food resources for developing new products and prefer more globalized food commodities, such as soybeans. Jicaro seeds might be used as raw material to develop innovative processed foods, such as a vegetable milk with very interesting sensory properties that would be of great interest to people allergic to lactose.

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FIGURES

Figure 1. Anatomy of jicaro seed (*Crescentia alata*).

Figure 2. Separation of jicaro seed proteins via RP-HPLC (A) followed by 15% SDS-PAGE of the resolved peaks (B). The bands with positive identification are labelled with red circles in B. “M” in figure 4B is the MW marker.

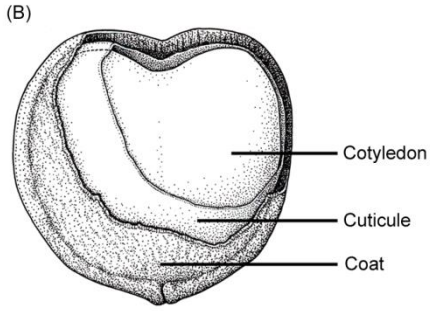
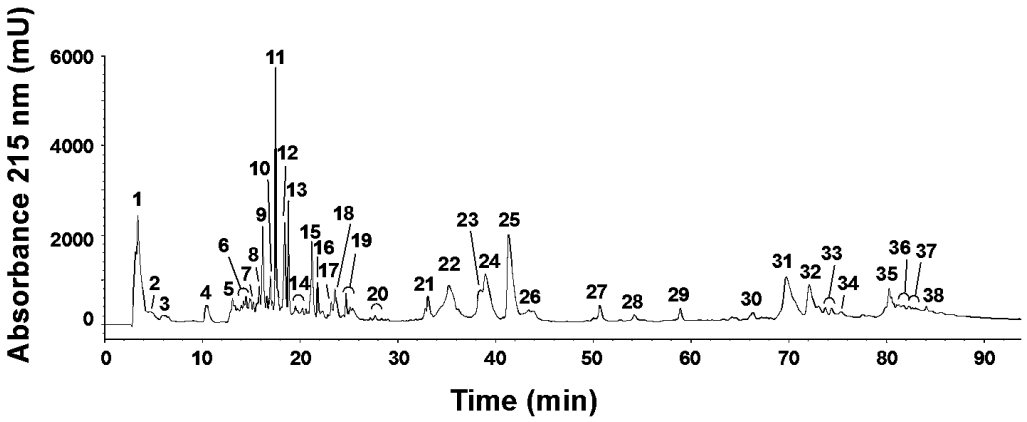


Figure 1.

(A)



(B)

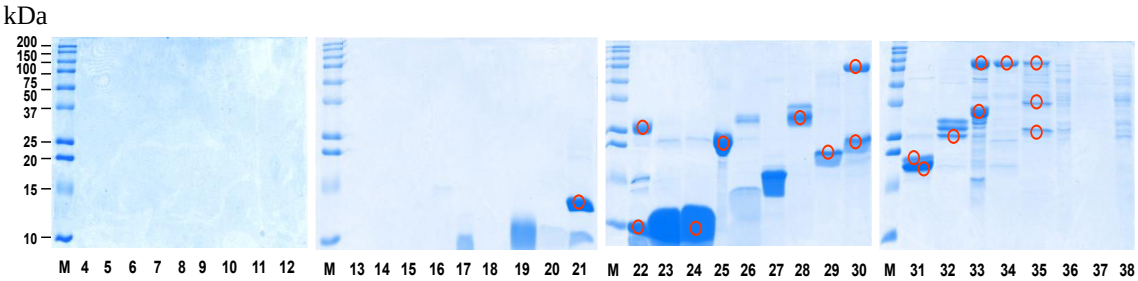


Figure 2.

Table 1. Main chemical composition of jicaro seed

Chemical composition	Jicaro seed			Soybean
	Seed	Cotyledon	Coat	
Proteins (g/100 g)	33.4±0.4	43.7±1.2	15.8±0.7	42.0±0.3
Lipids (g/100 g)	32.8±0.11	38.1±0.2	4.18±0.38	19.8[d]±0.8
Sugars (*g/100 g*)				
Sucrose	5.15±0.03	5.5±0.03	2.04±0.78	0.94-3.2[a]
Fructose	0.42±0.02	0.50±0.05	0.11±0.06	0.06-0.2[a]
Galactose	0.06±0.00	-	0.04±0.02	0.007-0.02[a]
Glucose	0.03±0.00	0.01±0.01	0.04±0.01	0.068-0.11[a]
Stachyose	0.11±0.01	0.14±0.01	<0.10	3.43±0.12
Raffinose	<0.10	<0.10	<0.10	6.62±0.02
Verbascose	<0.10	<0.10	<0.10	0.13±0.01
Fiber (*g/100 g*)				
Neutral detergent fiber (NDF)	23.6±0.4	13.8±0.2	71.2±1.0	11.4±0.2
Cellulose	7.3±0.3	3.3±0.1	34.2±0.6	2.6±0.1
Hemicellulose	8.8±0.4	8.3±0.2	19.4±1.0	5.9±0.2
Lignin	7.5±0.03	2.2±0.01	17.6±0.01	2.9±0.01
Minerals (*g/100 g*)				
Phosphorus	0.93±0.01	1.24±0.01	0.48±0.02	0.61±0.01
Potassium	0.55±0.01	0.71±0.01	0.35±0.01	1.97±0.02
Magnesium	0.41±0.003	0.52±0.01	0.23±0.01	0.23±0.003
Calcium	0.06±0.002	0.05±0.02	0.11±0.03	0.18±0.004
Sulfur	0.35±0.01	0.45±0.01	0.21±0.01	0.04±0.003
Sodium	0.01±0.001	0.03±0.03	0.01±0.0003	0.01±0.002
Trace minerals (mg/kg)				
Iron	159.4±4.4	90.9±4.1	228.9±48.5	55.5±2.2
Zinc	72.7±2.2	117.4±42.1	43.8±1.3	46.0±0.9
Copper	31.6±4.3	43.5±6.0	22.2±0.2	16.4±0.7
Manganese	24.7±0.4	32.1±8.4	35.9±0.9	27.2±0.9
Others micro - constituents				
Pythic acid (mg IP6/100 g d.w.)	1325±72.5	1032±61	486±53	1760[c]±8.0
Polyphenols (mg GAE/100 g d.w.)	0.49±0.08	0.33±0.03	0.24±0.03	268[b]±47
Tannins (mg TAE/100 g d.w.)	0.16±0.01	0.15±0.01	0.15±0.01	93[b]±20
γ -Tocopherols (mg/kg d.b)	11.3±0.13	8.82±0.02	nd	15.92±0.23
δ/β-Tocopherols (mg/kg d.b)	1.06±0.14	1.15±0.72	nd	2.84±0.04
α-Tocopherols (mg/kg d.b)	nd	0.45±0.03	nd	5.45±0.06

Results are expressed in dry weight basis as means ± standard deviations of three replicates
nd: not determined
[a](Song et al., 2013)
[b](Malenčić et al., 2012)
[c](Pedrosa et al., 2012)
[d](El Tinay et al., 1989)

Fatty acid composition (percentage of total fatty acids) of jicaro seed

	Jicaro			Soybean[a]
	Seed	Cotyledon	Coat	Cotyledon
Palmitic acid (16:0)	14.9±0.55	15.3±0.57	15.1±0.24	10.3±0.02
Stearic acid (18:0)	5.4±0.36	5.2±0.09	6.07±0.22	4.3±0.04
Arachidic acid (20:0)	0.74±0.13	0.70±0.03	0.82±0.07	-
Behenic acid (22:0)	0.33±0.10	0.29±0.03	0.38±0.02	-
Lignoceric acid (24:0)	0.24±0.08	0.22±0.08	0.30±0.04	-
Saturated fatty acids	21.61	21.71	22.67	
Palmitoleic acid (16:1, n-7)	0.45±0.02	0.50±0.02	0.57±0.04	-
Oleic acid (18:1, n-6)	55.3±0.93	56.5±0.70	54.1±0.57	22.2±0.61
Linoleic acid (18:2, n-6)	19.5±0.88	18.3±0.08	19.7±0.53	52.5±0.36
α-Linolenic acid (18:3; n-3)	2.4±0.23	2.3±0.03	2.4±0.12	7.3±0.12
Unsaturated fatty acids	77.65	77.60	76.77	

Results are expressed in g/100g oil as means ± standard deviations of three replicates.
[a](Slavin, 2003).

Table 3. Amino acid composition of jicaro seed

	% Composition			
	Jicaro			Soybean
	Seed	Cotyledon	Coat	Cotyledon
Essential amino acids (EAA)				
Leucine	7.2±0.45	7.4±0.10	7.4±0.2	8.0±0.07
Phenylalanine	5.2±0.30	5.4±0.2	4.6±0.10	5.4±0.007
Valine	3.9±0.95	4.2±0.3	5.3±0.50	4.9±0.04
Histidine	3.3±0.66	2.9±0.1	3.1±0.01	2.8±0.0004
Threonine	3.1±0.15	3.1±0.1	3.2±0.1	3.7±0.07
Isoleucine	3.0±1.04	3.2±0.3	4.3±0.8	4.7±0.01
Lysine	2.4±0.12	2.5±0.1	3.0±0.7	6.3±0.21
Methionine	1.8±0.28	1.8±0.2	1.6±0.9	1.3±0.002
Total EAA	29.9	30.6	32.6	37
Non-essential amino acids (NEAA)				
Glutamic acid	22.2±1.51	22.0±0.5	20.4±0.4	19.2±0.09
Tyrosine	4.5±1.27	3.8±0.1	4.0±0.1	4.0±0.05
Arginine	14.8±0.35	16.3±0.3	12.7±0.4	8.1±0.26
Aspartic acid	8.0±0.66	7.7±0.01	7.9±0.3	11.8±0.14
Glycine	5.4±0.47	5.0±0.04	6.3±0.2	4.4±0.04
Alanine	4.7±0.39	4.6±0.01	4.9±0.1	4.5±0.05
Serine	4.4±0.77	4.2±0.3	4.1±0.2	4.7±0.12
Proline	4.2±0.40	3.9±0.2	4.5±0.1	5.3±0.01
Cysteine	2.0±0.56	1.9±0.4	2.6±0.04	0.9±0.07
NEAA	70.1	69.4	67.4	63
EAA/NEAA	0.42	0.44	0.47	0.58

Results are expressed in dry weight basis as means ± standard deviations of three replicates.

Table 4. Protein matches obtained from peptide sequences of fractionated jicaro seeds via MALDI-TOF-TOF mass spectrometry.*

HPLC peak	Mol wt (kDa)	Peptide ion		Amino acid sequence	Protein match; species	Conf (%)	Sc	Related protein accession code[a]
		m/z	z					
21	14	1623.9 1497.7	1 1	E[ca]EEGVEIDESKFR EQLGTEGYQQMGR	Embryonic-like protein GEA6; *Arabidopsis thaliana*	99.0 99.0	9 16	~ Q02973
		1339.7	1	SLEAQQ[da]HLAEGR	EMB-1 protein; *Daucus carota*	99.0	20	~ P17639
22a	26	1192.6	1	QTDEYGNPIR	Dehydrin DHN1; *Avicennia marina*	99.0	11	~ A8CVF3
24	11	2032.1	1	RALAALIRP[ox]SSHQQRRR	Uncharacterized protein; *Glycine max*	97.6	7	~ XP_003546585
		768.4	2	RGEEQCQCEALR	**2S albumin seed storage protein**; *Juglans nigra*	man	man	~ AAM54365
25	23	1167.7	1	RTRQPPEKR	Predicted protein; *Physcomitrella patens*	man	man	~ XP_001755540
28	32	1732.9	1	DGNGFITAAELAHSMAK	Ca^{2+}-binding protein CML17; *Arabidopsis thaliana*	95.6	9	~ Q9LQN4
29	19	1613.9 2143.2 2271.3 2951.7	1 1 1 1	VDWRETPEAHVFK EEVKVEVEDGNILQISGER KEEVKVEVEDGNILQISGER ADVPGLKKEEVKVEVEDGNILQISGER	17.4-kDa class I heat shock protein; *Arabidopsis thaliana*	99.0 99.0 99.0 99.0	9 17 18 14	~ P19036
		2512.5	1	ASMENGVLTVTVPKEEVKKPEVK	17.8-kDa class I heat shock protein; *Solanum lycopersicum*	99.0	20	~ AAD30453
30a	96	1243.6 1274.6	1 1	AGEYKDYAAQK TGEYKDYAAEK	Embryonic protein DC-8; *Daucus carota*	99	16	~ P20075
30b	20	1801.9	1	QEEDDFFTSEGN[da]VQR	G patch domain-containing protein 8 isoform X3; *Musa acuminata*	99.0	9	~ M0T1Q6
		999.6	1	AGVLENIKR	Hypothetical protein 231423; *Selaginella moellendorffii*	96.4	7	~ XP_002970024
31a	18	1808.1	1	ETPEAHVFK[fo]ADLPGLR	17.6-kDa class I heat shock protein; *Arabidopsis thaliana*	99.0	15	~ P13853
31b	17	1931.1 974.6	1 1	ASMENGVLTVTVPKEEVK FRLPENAK	17.5-kDa class I heat shock protein; *Glycine max*	99.0 74.1	7 9	~ P04794
		1110.6	1	SLIPSFFSGR	18.2-kDa class I heat shock protein; *Vitis vinifera*	99.0	12	~ XP_002281285
		1794.1 1641.9	1 1	ETPEAHIFKADLPGLR IDWRETPEAHIFK	Predicted protein; *Physcomitrella patens*	99.0 99.0	14 8	~ A9SJI0
32c	30	1211.7 1452.9	1 1	LSFLYPGTTGR LKLSFLYPGTTGR	Uncharacterized protein; *Physcomitrella patens*	99.0 99.0	12 14	~ A9RJ06
		1105.7	1	I[pa]DTNGVIMR	Transcription repressor protein-related; *Musa balbisiana*	99.0	11	~ ABF70137
		2121.1	1	PGLTLGDVVPDLELDTTHGK	1-Cys peroxiredoxin B; *Oryza sativa*	99.0	11	~ P0C5D0
33a	110	1788.0	1	GAEMTNKEQNAKNHSK	Uncharacterized protein; *Selaginella moellendorffii*	99.0	10	~ D8TFU6
		1341.7	1	AALLLQ[da]ELGLSGR	Hypothetical protein I004822; *Eucalyptus grandis*	95.6	11	~ KCW54529

		1358.8	1	ELRATGELENAR	Cellulase-like protein; *Arabidopsis thaliana*	Masc	28	~ CAB83158
		1230.7	1	MASNLLKALIR	NADH dehydrogenase (ubiquinone) Fe-S protein; *Arabidopsis thaliana*	Masc	27	~ NP_566191
		1492.8	1	ETEMLESLTRKR	Uncharacterized protein; *Musa acuminata*	96.4	9	~ M0TCX2
33b	35	2041.1	1	AVGYCFASEGATVAFTYVK	Glucose and ribitol dehydrogenase homolog 1; *Arabidopsis thaliana*	99.0	12	~ Q9FZ42
		1263.7	1	P[ca]SATQVNNYGR	Predicted protein; *Populus trichocarpa*	95.2	10	~ XP_002304281
34	110	1358.8	1	CPQLEELSLNR	Uncharacterized protein; *Physcomitrella patens*	99	13	~ A9TQ22
		1326.8	1	AREAASRELPVK	Predicted protein; *Physcomitrella patens*	97.2	9	~ A9S3R4
		1239.5	1	FGSVQSPSSSTR	Thioredoxin Y2 protein; *Arabidopsis thaliana*	Masc	30	~ NP_175021
35a	110	1680.9	1	NAVVTVPAYFNDSQR	Heat shock 70-kDa protein; *Zea mays*	99.0	9	~ P11143
35b	40	1260.7	1	IWCTNLAPER	Aldose reductase; *Hordeum vulgare*	99.0	12	~ P23901
35c	27	1927.1	1	HFVAVHGVGHGAWVYYK	Alpha/beta hydrolase fold superfamily; *Gentiana triflora*	99.0	25	~ A5A7N5

* Cysteine residues determined in MS/MS analysis are carbamidomethylated. MW: SDS-PAGE apparent mass, in kDa. Confidence (Conf, %) and score (Sc) values were calculated by ProteinPilot® v. 4.0 using Paragon® algorithm. Matches identified by MASCOT public search engine are indicated with probability values provided by the server. Possible but unconfirmed or ambiguous amino acid modifications suggested by the automated identification software are shown between brackets with the following abbreviations: [ox]: oxidized; [da]: deamidated; [fo]: formylated; [pa]: propionamide; [ca]: carbamidomethylated. Man: *de novo* amino acid sequencing by manual spectrum interpretation.
[a] according to InterPro protein sequence analysis and classification (EMBL-EBI) at www.ebi.ac.uk.