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Caferana seeds (*Bunchosia glandulifera*) as a new source of nutrients: Evaluation of the proximal composition, solvent extraction, bioactive compounds, and δ-lactam isolation

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ABSTRACT

The proximal composition, amino acid, carbohydrate, and volatile profiles of caferana (*Bunchosia glandulifera*) seeds flour were here assessed. Seeds were also subjected to the following extraction processes: one with pressurized ethanol (PLE) and two with ethanol + supercritical CO_2 mixture at different temperatures and pressures (SC1 and SC2). Extracts were characterized in terms of caffeine, total phenolic, and δ-lactam. The characterization of caferana seed and its extracts is unprecedented in terms of carbohydrate and volatiles profiles, besides the δ-lactam identification/isolation. SC2 extract exhibited a higher caffeine (9.3 mg/g) and δ-lactam (29.4 mg/g) content, whereas the PLE extract contained a higher total phenolic amount (3.0 mgGAE/g). Caferana is regionally associated to protective effects on mental health. Its byproduct (seed) revealed to be a promising source of bioactive compounds, and a potential raw material of nutritive extracts and flours that can be incorporated into pharmaceutical, nutraceutical, cosmetic, and food products.

Introduction

Fruit processing industries produce substantial amounts of agro-industrial wastes worldwide. Depending on the variety, such residues can reach from 40 to 70% of total of fruits weight (Santos et al., 2020). Nowadays, efforts to use by-products as alternative materials for the preparation of flours, powders, and nutritional concentrates to be addition into various types of food have shown a notable increasing (Rodrigues et al., 2019). Besides that, the extraction of bioactive compounds with high added value has become a reality within the biorefinery concept (Banerjee et al., 2017).

Caferana (*Bunchosia glandulifera*) is a typical fruit species from the south region of Brazil, from which small fruits with sweet, red, and slightly spicy pulp are cropped. The pulp is a natural source of bioactive

Abbreviations: PUFAs, polyunsaturated fatty acids; GRAS, generally recognized as safe; SFE, supercritical fluid extraction; PLE, pressurized liquid extraction; CO_2, carbon dioxide; $ScCO_2$, supercritical carbon dioxide; M%, moisture, in percentage; VM%, volatile + moisture content, in percentage; GAE, gallic acid equivalent; TPC, total phenolic compounds; EDTA, ethylenediamine tetra-acetic acid; HPLC, high performance liquid chromatography; GC–MS, gas chromatography coupled to a mass spectrometry; HS-SPME, headspace solid phase microextraction; FTIR, Fourier transform infrared spectroscopy; NMR, nuclear magnetic resonance; IUPAC, International Union of Pure and Applied Chemistry Extraction; SC1, supercritical carbon dioxide and ethanol extraction (process 1); SC2, supercritical carbon dioxide and ethanol extraction (process 2); PLE, pressurized ethanol extraction (process 3).

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compounds with antioxidant potential, containing also a high concentration of minerals, polyunsaturated fatty acids (PUFAs), and carotenoids (Blank et al., 2017; Carvalho et al., 2019). Caferana fruit contains one or two seeds of a high volume, reaching up to 40% of total fruit weight. While the pulp is used by familiar agroindustry for jellies and juices production, the seed is discarded or donated to people who benefit for their own consumption after artisanal roasting and grinding. However, studies indicate that caferana seeds may contain bioactive compounds as caffeine, fatty acids, tocols, minerals, and exhibits an antioxidant activity due to the presence of phenolic compounds. Complementarily, there are popular reports suggesting that the consumption of roasted and grounded caferana seeds promote physical, mental well-being, and longevity effects (Fraga et al., 2020; Peixoto et al., 2017).

As has been done with several other types of fruit seeds and vegetables, more detailed studies of the chemical composition and nutritional potential of caferana seeds may be vital to indorse its use as raw material in the production of concentrated extracts and highly nutritional products with a beneficial action on the human organism (Maqsood et al., 2020).

For this purpose, the use of solvents *Generally Recognized as Safe* (GRAS) must be considered within the "*green*" extraction concept, as they promote the isolation of compounds with a high purity degree without solvent contamination, besides to be considered ecologically correct and commercially viable (Fraga et al., 2020; Viganó et al., 2016). Extractions with pure supercritical carbon dioxide or added ethanol as a co-solvent have been widely employed in several types of applications, including conditions for a selective extraction of compounds of interest to obtain either concentrated extracts or isolated compounds with a high purity degree. Such compounds display a potential application in pharmaceutical, nutraceutical, and food products (Banerjee et al., 2017).

Ethanol is the main solvent used to extract bioactive compounds from several vegetable matrices, including phenolic compounds, either by conventional methods or by pressurized liquid extraction (PLE). The advantages of applying PLE extraction are the higher extraction efficiency, lower solvent demand, reduced extraction time, enhanced extracts quality, possibility of a partial or total extract desolventizing due to the pressure reduction after extraction, among others (Viganó et al., 2016).

This study aimed to evaluate the caferana seeds as a potential source of nutrients and to attain bioactive-rich extracts. For that, the proximal composition of the raw material and its bioactive compounds profile were here assessed. Extracts were obtained from three different extraction conditions: two with supercritical carbon dioxide and ethanol as a co-solvent (SFE), and one by pressurized extraction with ethanol (PLE). Extracts were characterized in terms of caffeine and total phenolics. In addition to that, the molecule of δ-lactam, a nutritional compound rarely found in natural plant extracts was also identified.

Material and methods

Solvents and reagents

Carbon dioxide (CO_2, 99.0% purity) supplied by White Martins (Brazil) and ethanol (99.8% v/v) purchased from Synth (Brazil), were used as solvents in the extractions. The following chemicals were used to characterize the raw material and extracts: helium and nitrogen gas (99.0% purity, White Martins, Brazil); gallic acid standards (99.0% mass purity), caffeine standard (99.5% mass purity), amino acids (99.0% mass purity), α-aminobutyric (99.0% mass purity), carbohydrate standard (99.0% mass purity), triethylamine (99% v/v), phenyl isothiocyanate (99.0% mass purity) and phosphomolybdic acid (99.0% mass purity) (Sigma-Aldrich, USA), rice flour protein standard (batch 70501-A, VELP, Brazil), anhydrous sodium carbonate (99.5% mass purity), sodium acetate (99.5% mass purity), ammonium hydroxide (97% mass purity) (Exodus, Brazil), Folin-Ciocalteu reagent (Dynamics, Brazil), methanol (99.9% v/v), hexane (98.5% v/v), acetonitrile (99.9% v/v), ethyl acetate (99.9% v/v), deuterated chloroform (99.9% v/v), and chromatographic plate covered with silica gel (60, F254) (Merck, Germany), silica gel 60A (0.035–0.070 mm. Across organics™, Fisher Scientific. Switzerland), ultrapure water (Millipore direct-Q3 UV, Millipore Corporation, USA).

Characterization of the raw material and proximal composition

The fruits were collected between January and April 2019 in the city of Santo Antônio da Patrulha, Rio Grande do Sul State, Brazil. Seeds were manually separated from the pulp, then sanitized with fresh water, separated from the peel, and thus dried in a lyophilizer apparatus (Liobras, model L101, Brazil) at $T = (-50 \pm 1)$ °C and $p = (16 \pm 2)$ μHg during 24 h. After drying, seeds were grounded in a refrigerated knife mill at $T = (15 \pm 1)$ °C (Solab, model SL35, Brazil). The material was weighed on an analytical balance with an accuracy of ± 0.0001 g (Precisa XT 220A, Switzerland), packed into plastic bags, and stored in an ultra-freezer (Model Glacier™, Thomas Scientific, U.S.A.) at $T = (-78 \pm 2)$ °C until its utilization.

The lyophilized raw material was characterized in terms of humidity (M%) by Karl Fischer titration in a KF Titrino Plus device (model 870, Metrohm, Switzerland) equipped with a Thermoprep KF oven (model 832, Metrohm, Switzerland) according to AOCS Ca 23–55 method (AOCS, 2017). Total volatiles and moisture content (VM%) was assessed by the gravimetric method, following the AOAC 930.04 procedure (AOAC, 2013). For total lipid content, samples were dried in a ANKOM dryer system (RDI Dryer, ANKOM Technology, U.S.A.) and further submitted to an extraction with hexane in a ANKOM oil/fat extractor (XT-15 Extractor, ANKOM Technology, USA), according to the official procedure Am 5–04 AOCS (AOCS, 2017). Total nitrogen content was appraised by the combustion method using a Dumas VELP NDA equipment (model 701, Scientifica, Brazil), in which the protein amount was estimated by multiplying the total nitrogen content by a correction factor (6.25), being that such results were interpreted in the DUMASoft™ software. The proximal composition analysis (ashes, soluble solids, fibers) and pH were carried out according to the analytical methods adopted by the Adolfo Lutz Institute (IAL, 2008). Finally, total carbohydrate content was determined by mass difference, i.e., total carbohydrate = 100 − (moisture + ash + proteins + lipids + fibers).

Carbohydrates profile by ion chromatography

The carbohydrate profile of the raw material was assessed by ion chromatography according to a procedure adapted from the literature (Blanco et al., 2004; Pico et al., 2015). Carbohydrates were obtained in exhaustive aqueous extraction with (5 ± 0.2) g lyophilized seeds and crushed into 150 mL deionized water under agitation for 2 h at $T = (60 \pm 2)$ °C. After extraction, solutions were lyophilized during 36 h at $T = (-50 \pm 1)$ °C and $p = (16 \pm 2)$ μHg (Liobras, model L101, Brazil) and stored into amber glass vessels which, in turn, were placed inside a desiccator. Sampling was performed by adding (20.0 ± 0.1) mg extract diluted in 10 mL ultra-pure Milli-Q water. Analyzes were performed on an ion chromatograph (Metrohm, Herisau, Switzerland), composed of a 940 Professional IC Vario unit, with isocratic pump, an 858 Professional Sample Processor carousel with an ultrafiltration system, and a Pulsed Amperometric Detector (PAD) within the range from −250 to 900 mV.

The compounds separation was performed inside a Hamilton RCX-30 column (250 mm length, 4.6 mm i.d., Metrohm, Herisau, Switzerland) within a pH range from 1 to 13. A pre-column Guard RP3 HC/4.0 (Metrohm, Herisau, Switzerland) with the same stationary phase as the chromatograph column was inserted before the main column. Both column and pre-column were placed inside an oven with a controlled temperature of 32 °C, whereas PAD was set at 45 °C. NaOH 20 mmol/L was used as eluent at a flow rate of 0.5 mL/min with a sample injection

volume of 20 μL, totalizing 75 min run time per sample.

Amino acids profile

Total amino acids amount was assessed by reverse phase column chromatography in a high-performance liquid chromatograph (HPLC) following the methodologies of Hagen et al. (1989) and White et al. (1986). To attain the total amino acid composition, (50 ± 2) mg raw material was subjected to acid hydrolysis with phenol. Alpha-aminobutyric acid was added to the released amino acids as an internal standard. Acids were removed by evaporation $T = (50 \pm 2)$ °C under vacuum (p = 700 mmHg) (Marconi, MA-1220, Brazil), and a re-evaporation was executed adding a solution of methanol, triethylamine, and water (2:2:1, in volume) to the sample. The hydrolysate derivatization was done with a methanol, triethylamine, ultrapure water, and phenyl isothiocyanate (PITC) solution (7.5:1:1:0.5, in volume). Subsequently, amino acids were dissolved in pure acetonitrile before the HPLC analysis. Samples were introduced into the HPLC column (LUNA C18 100 A 5 μm × 250 mm × 4.6 mm 00G-4252-EQ), accoupled to a pre-column (C18, 4 mm × 3.0 mm, or Shim-Pack CLC G-ODS 4) with monitoring at a wavelength of 254 nm. The mobile phase was a 140 mM sodium acetate buffer solution (pH 6.0) containing 0.05% triethylamine (eluent A) and acetonitrile/water (60:40, v/v, eluent B). Amino acids were then identified by their retention times and quantified based on their peak areas by external standardization, using calibration curves constructed for 17 amino acids in different concentrations (1.5, 7.5, 15, 30, 120, 300, 600 μmol/L), diluted in a HCl (0.1 mol/L) solution.

Volatile compounds profile

The qualitative analysis of the volatile fraction was carried out by the solid phase microextraction (HS-SPME) method in a gas chromatograph coupled to a mass spectrometer (GC–MS). For that, (2.0 ± 0.1) g liquid sample were placed inside a 20 mL flask, in where 5 mL distilled water was added, and the flask was thus sealed with a silicone septum and placed inside a thermostatic bath at $T = (70 \pm 2)$ °C during 30 min to volatiles releasing. Then, a PMDS-DVB fiber (polydimethylsiloxane-divinylbenzene, 65 μm) was carefully introduced through the silicone septum in the headspace region to adsorb the analytes present in the vapor phase. After 30 min the fiber was removed and directly inserted into the GC–MS injector (T = 220 °C) to release the volatiles inside the apparatus.

To identify the volatiles, an HP/Agilent gas chromatograph (model 6890 Series GC System) was used, coupled to a mass spectrometer (Hewlett Packard 5973 Mass Selective Detector) operating with a 70-eV electron impact ionization source and an HP5-MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, USA). The carrier gas used was Helium with a flow rate of 1.00 mL/min. The column temperature was programmed in a ramp from 35 °C to 215 °C at 3 °C/min. The detector temperature was 250 °C. Peak identification was performed by comparing the retention times of *n*-alkane patterns (C8-C40) under the same analysis conditions and from the mass spectra compared to the NIST 2.0 electronic library, assuming a similarity higher than 90%.

Experimental extraction assays

Experimental extractions were carried out in a pilot extractor unit (ExTrAE Laboratory, UNICAMP, Brazil). The extractor vessel was packed with (5.37 ± 0.10) g powdered seeds, which were previously prepared and standardized in terms of particle size (181.39 ± 1.80 μm). Glass spheres were inserted into the vessel until its completely volume filling. Extracts were obtained in three process conditions, described as follows: (i) supercritical carbon dioxide as solvent and ethanol as a co-solvent (90:10 w/w), at $T = (60 \pm 1)$ °C and $p = (400 \pm 5)$ bar (SC1); (ii) supercritical carbon dioxide as solvent and ethanol as a co-solvent (90:10 w/w), at $T = (70 \pm 1)$ °C and $p = (180 \pm 5)$ bar (SC2); (iii) pressurized anhydrous ethanol as solvent at $T = (60 \pm 1)$ °C and $p = (400 \pm 5)$ bar (PLE). Temperature was controlled by a thermostatic water bath, whereas pressure was adjusted by pumping the solvent through a pressure valve.

A period of 30 min was adopted for the system stabilization, ensuring that both temperature and pressure conditions were attained, as well as the packaged raw material was completely soaked by the solvents. Extractions CS1 or CS2 were thus started by flowing the solvent ($ScCO_2$ and ethanol) at 2.51×10^{-3} kg/min flow rate though the bed with a solvent to feed mass ratio S/F = 75.5. For the PLE extraction, ethanol was pumped at 3.95×10^{-4} kg/min flow rate with S/F = 13.26.

All extraction assays occurred in a single batch along 180 min and in triplicate. After that, the remained solvent in extracts were completely removed by evaporation under vacuum (p = 700 mmHg) at $T = (40 \pm 1)$ °C. Dried extracts were stored inside amber glass vessels, weighed, and preserved in an ultra-freezer at $T = (-78 \pm 2)$ °C until analysis.

Nutritional evaluation of extracts

Caffeine content

Caffeine content was assessed in a high-performance liquid chromatography (HPLC), according to the method described by Cordeiro (2009). A Dionex HPLC liquid chromatograph (UltiMate 3000 Standard LC, U.S.A.) with UV–Vis diode array detector (DAD) at a wavelength from 270 to 280 nm was used with mobile phases methanol (A) and ultrapure water (B) in the proportion of 70/30 (v/v). Extract samples were dissolved in ultrapure water for 10 min in an ultrasonic bath (USC-2800[a] ultrasonic bath, freq. 40 kHz, pot. 154 W, UNIQUE, Brazil) before injection. 20 μL diluted sample was injected into a C18 reverse phase column (Infinity Lab Poroshell 120 EC-18 4.6 × 100 mm × 2.7 μm; Agilent Technologies, USA). Caffeine content was quantified by external standardization assuming calibration curves built in triplicate within caffeine concentration range from 0 to 100 ppm.

Total phenolic compounds content

Total phenolic compounds (TPC) content was quantified according to the procedure described by Singleton et al. (1999). The method is based on the alkalinity reduction of the reagent Folin-Ciocalteu (phosphomolybdic acid + sodium tungstate) by phenols into molybdenum, promoting a blueish color. Sample absorbance was measured at a wavelength of 760 nm in a UV–Vis spectrophotometer (Thermo Scientific™ Orion™ AquaMate 8000 UV–Vis, Thermo Fischer Scientific, USA). TPC content was calculated using a calibration curve built with a gallic acid standard (0.25–8 ppm) and expressed as gallic acid equivalents (mgGAE/g). The analysis was performed in triplicate.

Separation, purification, identification, and quantification of δ-lactam 1,6-dihydropyridin-2(3H)-one (1)

The following analyzes were performed for the separation, purification, identification, and quantification of the precipitated crystal **found** in the **extracts**: separation and purification was by optical microscopy (Leica DM2700 M, China); thin layer chromatography (TLC) (Alugram Sil G, Macherey Nagel, Germany); column chromatography on silica (CCS) (Across organicsTM, Fischer Scientific. Switzerland).

Identification of the precipitated isolate by gas chromatography-mass spectrometry (GC–MS) analyses were performed in an Agilent® 6890 Series GC system coupled to an HP 5973 (Agilent Technologies, USA) mass spectrometer with an electron ionization source (EI) operation at 70 eV, equipped with HP 5-MS silica capillary column (30 m × 0.25 mm ID × 0.25 m film thickness), using helium as the carrier gas (1.0 mL/min) and a split ratio of 1:25. The injector and detector temperatures were maintained at 260 °C and 280 °C, respectively. Column temperature was kept at 50 ∘ C for 1 min, increased to 290 °C at a rate of 20 °C/min, totalizing 20 min of analysis run. One microliter of a 1 mg/

mL solution was injected.

Identification of the precipitate isolate by NMR spectra were recorded in an Advance spectrometer at 600 (Advance III 600 MHz, Bruker, USA) (1H NMR) and 150 (13C NMR) MHz. Chemical shifts were reported in parts per million (ppm). Identification of the precipitate isolate was also confirmed by Fourier transform infrared spectroscopy (FTIR) (ATR Cary 630 Agilent, Agilent Technologies, USA). Determination of the melting point by MP900 Melting Point system (Mettler Toledo, Brazil) equipped with a glass capillary, remaining in analysis for a period of 60 min. The quantification of δ-lactam 1 was performed on HPLC (UltiMate 3000 Standard LC, USA) assuming the alkaloids methodology, adapted from Cordeiro (2009). Analyzes were performed at the LaBioChem Laboratory of the Research Group of Prof. Anita J. Marsaioli at the Institute of Chemistry, UNICAMP (Brazil).

Statistical analysis

Statistical analysis of the experimental data was executed by the software Statistica® 7.0 (Statsoft, USA). Results were expressed as the standard mean of triplicates and evaluated by analysis of variance (ANOVA). The Tukey test was thus assumed to evaluate the significative differences between the means at a confidence level $\alpha = 0.95$.

Results and discussions

Characterization of the raw material and proximal composition

To assess the raw material qualities, lyophilized caferana seeds were subjected to a complete characterization (Table 1) that included composition in volatiles and moisture, ash, fibers, total lipids, total proteins, total carbohydrates, soluble solids, besides pH.

Results expressed as mean ± standard deviation. %w/w in dry basis.

A difference of 3.3% between volatiles + humidity (VM%) and humidity (M%) may be explained by the evaporation of other volatile compounds besides the water contained in caferana seeds. The same was noted by Monroy et al. (2016) when measuring the moisture of vegetable raw materials by Karl Fischer and gravimetric methods. Caferana seeds contain high levels of proteins and total carbohydrates, the latter was confirmed by the soluble solids analysis (°Brix).

Experimental results found for ash, fibers, and total lipids contents, as well as pH are close to those reported by Blank et al. (2017). On the other hand, values for proteins and soluble solids here quantified differed from those reported by Blank et al. (2017) who found 7% and 4.52%, respectively. Differences may be associated with several aspects, including harvesting procedure, nutrients composition of soil, local climate, rainfall and solar incidence, storage, transport, and so on. For comparison purposes, a flour made by the byproduct (seeds, peels, and pulp) of "camu-camu" (*Myrciaria dubia* (HBK) McVaugh), that is considered a Brazilian superfruit, has a protein content from 6.86 to 7.04% (Santos et al., 2022). This composition is relatively lower than the one found in the caferana seeds flour, which leads us to infer that caferana seeds have a high protein level in relation to other fruit seeds.

Table 1
Proximal composition (%) of caferana seeds.

Compound/property	Mean ± standard deviation (%)
Moisture (M)	8.4 ± 0.2
Volatiles + moisture (VM)	11.7 ± 0.4
Ashes	2.76 ± 0.05
Fibers	1.8 ± 0.1
Total lipids	0.47 ± 0.07
Total proteins	15.8 ± 0.3
Total carbohydrates	68 ± 1
Soluble solids (°Brix)	29.1 ± 0.5
pH	6.6 ± 0.1

Carbohydrates profile

The quantitative profile evaluation of carbohydrates and amino acids, in addition to the qualitative volatile profile, are unprecedent for caferana seeds. The relevance of such study concerns the possibility of assuming caferana seeds, which are treated up to date as a residue from caferana fruit, in processing products with potential human nutritional action, as a food or pharmaceutical additive, as has been done with other raw materials types as seeds and peels from fruits and vegetables (Maqsood et al., 2020).

Experimental outcomes for the carbohydrate content and profile are shown in Table 2. Sorbitol was the compound found with the highest concentration (265 ± 4 g/mg, corresponding to about 42.7% of total carbohydrates), followed by fructose (28.8%), arabinose (7.25%), and glucose (6.61%). Sorbitol is a polyol widely found in fruits as pears, plums, apples, among others. It exhibits properties that range from humectant action to antioxidant one, with the ability to sequester metals involved in an oxidative reaction. Sorbitol absorption in the human organism is slow, being thus recommended as a sweetener in diet products. Moreover, sorbitol is one of the polyols most used in the food industry due to its multiple functional characteristics (Rice et al., 2019).

About 49.7% of 620.6 mg/g carbohydrates found in caferana seeds is composed of reducing sugars (fructose, glucose, maltose, arabinose and xylose) that, along with proteins, can contribute to the flavor formation by the Maillard Reaction during the roasting procedure (Shibao & Bastos, 2011).

Amino acids profile

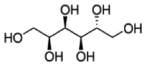
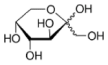
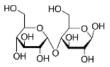
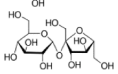
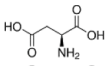
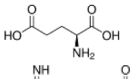
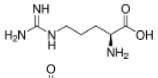
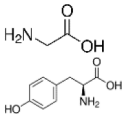
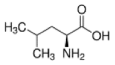
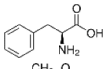
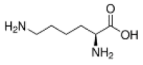
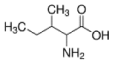
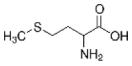
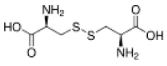
Amino acids composition found in caferana seeds is shown in Table 2. The average percentage of total amino acids (15.5%) present in the caferana seeds are in agreement with 15.7% protein shown in Table 1. Despite containing about 34.5% amino acids that are considered essential to the human organism (see Table 2), caferana seeds also exhibit expressive content of aspartate (16.2%), followed by glutamate (15.7%), arginine (9.86%), and leucine (6.04%). Such amino acids act as metabolic fuels and are involved in the regulation of neurological development and its functions (Wu, 2013). This outcome can thus contribute to the elucidation of testimonials of well-being, mental lucidity, and longevity described by caferana seeds consumer (Fraga et al., 2020; Peixoto et al., 2017).

Maillard reactions are one of the most important reaction involved in the roasting process of seeds and grains, as coffee and malt, being responsible for the flavor development. Such reactions occur with the presence of reducing sugars as xylose, arabinose, glucose, maltose, and fructose, along with amino acids such as lysine, arginine, phenylalanine, leucine, glutamic, and aspartic acid. The Maillard reaction depends on some factors, including the presence of transition catalyst metals Cu^{2+} and Fe^{2+}, raw material moisture from 30 to 70%, and temperatures above 40 °C (Shibao & Bastos, 2011). Moisture (~55%) and the presence of Cu^{2+}, and Fe^{2+} in caferana seeds *in nature* were evidenced by Blank et al. (2017), besides other minerals in high concentrations, as K, P, Ca, and Mg.

Volatiles profile

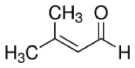
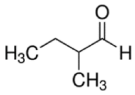
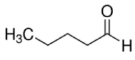
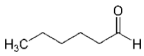
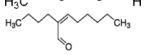
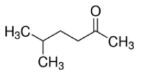
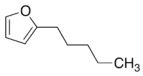
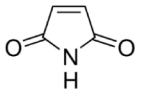
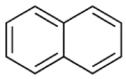
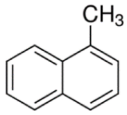
The volatile fraction of raw materials and products are mainly associated to their odor characteristics, since the human nose can assimilate the compounds diffused in the air (Gonçalves et al., 2018). The volatile profile was qualitatively assessed in a HS-SPME/GC–MS, which allowed the identification of 10 volatile compounds in the raw material (Table 3). Although the imide 1H-pyrrole-2,5-dione (maleimide) was the main compound found the volatile fraction of caferana seeds, no information about its odor description was found. However, such compound may rearrange the *N*-terminal of cystine (Cys) to generate thiazines (Gober et al., 2021). Thiazine nucleus is being studied

Table 2
Carbohydrates and amino acids profile of caferana seeds.

Compound class	Mean ± standard deviation (mg/g)	Composition (%)	Molecular mass (g/mol)[a]	Empirical formula[a]	2D Molecule[a,b]
Carbohydrates					
Sorbitol	265 ± 4	42.7	182.2	$C_6H_{14}O_6$	
Fructose	178.8 ± 0.7	28.8	180.2	$C_6H_{12}O_6$	
Arabinose	45 ± 1	7.25	150.1	$C_5H_{10}O_5$	
Glucose	41.0 ± 0.4	6.61	180.2	$C_6H_{12}O_6$	
Maltose	33.5 ± 0.2	5.40	342.3	$C_{12}H_{22}O_{11}$	
Inositol	32.16 ± 0.6	5.18	180.2	$C_6H_{12}O_6$	
Sucrose	14.5 ± 0.2	2.34	342.3	$C_{12}H_{22}O_{11}$	
Xylose	10.7 ± 0.5	1.72	150.1	$C_5H_{10}O_5$	
Total carbohydrates	620 ± 8	100			
Amino Acids					
Non-essentials					
Aspartate (Asp)	25.17 ± 0.03	16.2	133.1	$C_4H_7NO_4$	
Glutamate (Glu)	24.40 ± 0.02	15.7	147.1	$C_5H_9NO_4$	
Arginine (Arg)	15.32 ± 0.02	9.86	174.2	$C_6H_{14}N_4O_2$	
Alanine (Ala)	9.39 ± 0.02	6.04	89.1	$C_3H_7NO_2$	
Proline (Pro)	7.84 ± 0.01	5.05	115.1	$C_5H_9NO_2$	
Serine (Ser)	6.83 ± 0.01	4.40	105.1	$C_3H_7NO_3$	
Glycine (Gly)	6.42 ± 0.01	4.13	75.1	$C_2H_5NO_2$	
Tyrosine (Tyr)	6.36 ± 0.01	4.09	181.2	$C_9H_{11}NO_3$	
Essentials					
Leucine (Leu)	12.48 ± 0.04	8.03	131.2	$C_6H_{13}NO_2$	
Phenylalanine (Phe)	8.33 ± 0.01	5.36	165.2	$C_9H_{11}NO_2$	
Valine (Val)	8.27 ± 0.01	5.32	117.2	$C_5H_{11}NO_2$	
Lysine (Lys)	7.06 ± 0.01	4.54	146.2	$C_6H_{14}N_2O_2$	
Isoleucine (Ile)	6.79 ± 0.01	4.37	131.2	$C_6H_{13}NO_2$	
Threonine (Thr)	4.66 ± 0.01	3.00	119.1	$C_4H_9NO_3$	
Histidine (His)	3.02 ± 0.01	1.94	155.2	$C_6H_9N_3O_2$	
Methionine (Met)	2.23 ± 0.01	1.44	149.2	$C_5H_{11}NO_2S$	
Cystine (Cys)	0.82 ± 0.01	0.53	240.3	$C_6H_{12}N_2O_4S_2$	
Total amino acids	155.4 ± 0.3	100			

[a]From PubChem (2021);
[b]D, L, or DL-isomer.

Table 3
Qualitative profile of volatile compounds in caferana seeds.

t_R (min)	Component	relative %[a]	Molecular weight (g/mol)[b]	Empirical formula[b]	2D molecule[b]	Odor description[c]	VP (mmHg/ 25 °C)[b,c]
2.58	3-Methyl-2-butenal	1.05	84.12	C_5H_8O		Sweet, fruity, pungent, brown, nutty; with almond and cherry nuances	8.25 (est)
2.68	2-Methylbutanal	0.57	86.13	$C_5H_{10}O$		Musty, almond, cocoa, coffee, nutty; with malty and fermented nuances	49.32 (est)
3.08	Pentanal(valeraldehyde)	0.61	86.13	$C_5H_{10}O$		Fermented, bready, almond, bitter, nutty, coffee, chocolate, fruity; with and berry nuance	31.79 (est)
5.13	Hexanal(caproaldehyde)	21.32	100.16	$C_6H_{12}O$		Green, fatty, leafy, vegetable, fruity, clean; with woody and fresh green nuances	10.89 (est) – 11.3
29.72	2-Butyl-2-octenal	4.86	182.3	$C_{12}H_{22}O$		Green, fruity, pineapple, vegetable, sweet, ripe, juicy	0.011 (est)
8.11	5-Methyl-2-hexanone	1.02	114.19	$C_7H_{14}O$		Pleasant fruity	5.197 (est) – 5.77
12.28	2-Pentylfuran(2-amylfuran)	4.23	138.21	$C_9H_{14}O$		Fruity, green, floral, earthy, beany; with vegetable-like nuances	2.022 (est)
22.24	1H-pyrrole-2,5-dione (maleimide)	41.41	97.07	$C_4H_3NO_2$		Not described	
20.98	Naphthalene(naphthalin)	4.63	128.17	$C_{10}H_8$		Pungent; dry; resinous	0.159 (est) – 0.085
26.00	1-Methylnaphthalene	1.14	142.20	$C_{11}H_{10}$		Naphthyl chemical, medicinal; with camphor nuance	0.052 (est) – 0.067
Total identified		80.84					
7.43	N.I.	1.34					
8.48	N.I.	0.86					
11.87	N.I.	1.01					
12.02	N.I.	1.05					
22.27	N.I.	2.78					
28.38	N.I.	1.26					
30.66	N.I.	1.62					
35.12	N.I.	8.26					
38.06	N.I.	0.98					
Total unidentified		19.16					

[a]By internal standardization;
[b]From PubChem (2021);
[c]From The Good Scents Database (2021);
[d]Vapor pressure (The Good Scents Company, 2021).

as a potential pharmaceutical molecule to be employed in therapeutics treatments of Alzheimer's disease, cancer, diabetes, and others (Choudhary & Silakari, 2018). Maleimide is also a precursor of several other compounds with anti-fungal, anti-microbial, anti-bacterial, and anti-cancer actions (Salameh et al., 2020). These findings also may be associated with the longevity reported by caferana seeds consumers (Fraga et al., 2020; Peixoto et al., 2017).

Compounds belonging to the aldehydes class represent 28% volatile fraction. The odor of the main aldehyde hexanal (21.32%), is described as green, fatty, leafy, vegetable, fruity, clean, with woody and fresh green nuances, which is related to green grains. The presence of 2-pentylfuran may also contribute for the green odor. On the other hand, the aldehydes as 3-methyl-2-butenal, 2-methylbutanal, and pentanal may attribute almond, nutty, and fermented nuances.

Hydrocarbons (5.8%) as naphthalene and 1-methylnaphthalene may cause off-flavor nuances to the caferana seeds odor. About 19% of volatile compounds were not identified due either to its absence in NIST or in the internal standard. Such compounds may also exhibit or not an influence over the caferana seeds odor.

In general, the analysis revealed that the lyophilized caferana seeds odor is formed by at least five classes of compounds (aldehydes, imide, ketone, ether, and hydrocarbon). The mechanisms of formation of these compounds are associated with complex chemical reactions involving precursors as fatty acids, carbohydrates, amino acids, phenolic compounds, caffeine, among others (Marek et al., 2020). Also, a part of volatiles may be removed during the lyophilizing process.

Evaluation of extracts

Caffeine content

Caffeine is one of the most consumed alkaloids worldwide, being its consumption related to the possibility of increased alertness, stabilizing mood, cognitive performance, in addition of improving a long-term memory and preventing memory deficit caused by chronic

neurodegenerative diseases (Cunha & Agostinho, 2010). Caffeine is commonly found in expressive amount in natural products as maté leaves (*Ilex paraguariensis*), guarana seeds (*Paullinia cupana*), coffee seeds, and black tea leaves (*Camellia sinensis*), whereas its presence in fruit seeds are rare or inexpressive (Kopcak & Mohamed, 2005; Marek et al., 2020; Saldaña et al., 2002).

Results of caffeine content (in dry basis) in extracts are shown in Table 4, where is notable that the SC2 extraction led to a higher caffeine amount in the extract, followed by SC1, and PLE extractions lastly. The caffeine content found in the extracts of caferana seeds was close to those reported for maté leaves (*Ilex paraguariensis*), from 0.5 to 0.8% (Saldaña et al., 2002), and coffee seeds, 0.4 to 1.3% (Marek et al., 2020).

Studies carried out on the efficiency of CO_2 plus ethanol as a co-solvent, combined with heating, found an increased solvation power of caffeine and, consequently, increased extraction yields. However, the pressure increasing in the co-solvent ethanol had a retrograde effect, resulting in decreased yield values (Kopcak & Mohamed, 2005; Saldaña et al., 2002). The same effects were observed in this study. As shown in Table 4 higher yields were reached for SC2, in which $T = 70$ °C and $p =$ 180 bar, being 44.03% higher than for SC1 extraction, in which $T =$ 60 °C (lower temperature) and $p = 400$ bar (higher pressure).

Fraga et al. (2020) obtained similar results for the caffeine content in sequential extractions in caferana seeds, using supercritical CO_2, followed by PLE ethanol, under $T = 60$ °C and $p = 400$ bar. Under the same process conditions ($T = 60$ °C and $p = 400$ bar), extractions with only supercritical CO_2 resulted in an extract with 8.44 mg/g (Fraga et al., 2020), against 6.45 mg/g for SC1 (supercritical CO_2 plus ethanol at 90:10 w/w). Therefore, ethanol may have negatively affected the caffeine extraction. Note that, the caffeine content in the extract from PLE was the lower when compared with SC1 and SC2. Also, the increased pressure in SC1 may has changed the physical conditions of CO_2 in terms of polarity and viscosity (Saldaña et al., 1999), affecting thus its capacity of solubilizing caffeine.

Total phenolic compounds

Total phenolic compounds (TPC) in extracts are also available in Table 4, where is notable that PLE extract contained the highest TPC concentration, followed by SC2, and SC1. Results from PLE extraction are similar to the TPC value found in caferana seeds extracts obtained by conventional ethanol extraction procedure (Peixoto et al., 2017). Viganó et al. (2016) reported comparable results in passion fruit peel extracts obtained by pressurized ethanol under the same process conditions of T and p. TPC content in extracts SC1 and SC2 were higher than those obtained by Fraga et al. (2020) in extracts with pure supercritical CO_2.

Contrarily to caffeine, higher TPC values in extracts were reached when ethanol was used as a pure or as a co-solvent. Phenolic compounds may have a greater affinity for polar solvents as ethanol and water. Considering that ethanol can modify the physical conditions of CO_2, decreasing viscosity and increasing its polarity, the use of ethanol as a co-solvent in SC1 and SC2 extractions may have contributed to the increased capacity of extracting such phenolic compounds. Fraga et al. (2020) also reported low TPC content in extract from a first extraction run with supercritical CO_2 and a relevant TPC increasing in extracts from the second run with pressurized ethanol.

Table 4
Bioactive compounds composition of the caferana seed extracts.

Extract type	T (°C)	p (bar)	Caffeine (mg/g)*	Total phenols (mg_{GAE}/g)*	δ-Lactam **1** (mg/g)*
SC1	60	400	6.45 ± 0.07^{B}	1.76 ± 0.07^{C}	20.04 ± 0.38^{B}
SC2	70	180	9.29 ± 0.12^{A}	2.44 ± 0.08^{B}	29.36 ± 0.18^{A}
PLE	60	400	3.19 ± 0.05^{C}	3.01 ± 0.03^{A}	8.19 ± 0.01^{C}

*Different letters in the same column indicate statistically significant differences ($p < 0.05$). SC1: supercritical carbon dioxide and ethanol extraction (process 1); SC2: supercritical carbon dioxide and ethanol extraction (process 2); PLE: pressurized ethanol extraction (process 3).

Separation, purification, identification, and quantification of the compound δ-lactam 1,6-dihidropiridin-2(3H)-one (1)

The study of the separation, purification, identification, and quantification of the compound δ-lactam **1** from plant origin is unprecedented in the literature. Such compound is rarely found in plants, and its isolation was only reported as subunit of a series of alkaloids from *Gelsemium elegan* (heartbreak grass), *Piper arborescens*, and *Piper sintenense* (Albrecht et al., 2014; Chen et al., 2002; Moreira et al., 2018).

The solid-white structures formed during the extraction processes (Fig. 1a) were separately exposed on glass slides for optical microscopy evaluation and the confirmation of the crystalline structure formation. It can be seen in Fig. 1b and c that the solid formations have stick and needle drawings and also transmit a certain luminosity, giving indications of a true crystalline structure.

For the separation and purification, dried extracts that formed crystalline structures were solubilized in chloroform and subjected to a thin layer chromatography through a glass capillary dripped plates to define the best eluent to be used in the subsequent separation stage by column chromatography on silica. The eluent that exhibited the best separation power and, therefore, used as a mobile phase, was an ethyl acetate and methanol mixture (90:10 v/v). Fractions of interest were separated, and the solvent was then evaporated at $T = (35 \pm 1)$ °C under vacuum ($p = 700$ mmHg), resulting in a white solid. The isolated solid was solubilized in ethyl acetate and analyzed in GC–MS showing a single chromatographic peak, in 9.105 min retention time (Fig. 2a), indicating the probable purity of the isolated compound.

The mass spectrum (Fig. 2(b)) shows the molecular ion (M^+) with mass to charge ratio m/z 97 base peak of the spectrum. The fragments m/z 78.69 and 54 are also present in the spectrum. The characteristics are compatible with the compound δ-lactam 1,6-dihydropyridin-2(3H)-one according to IUPAC, of molecular formula C_5H_7NO, as shown in Fig. 2(b).

The Nuclear Magnetic Resonance (NMR) analysis was carried out to confirm the structure of δ-lactam **1**. Analysis was carried out in an NMR Spectrometer in which the compound was solubilized in deuterated chloroform ($CDCl_3$). Signals obtained in the hydrogen spectrum are in line with those reported in the literature by Assaf et al. (2014) and Baron et al. (2011) who obtained synthetic δ-lactam **1**. Signals between 0 and 2 ppm refer to impurities. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ 6.4 (br, 1H), 5.81–5.73 (m, 2H), 4.01–3.96 (m, 2H), 2.96–2.92 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$, ppm) δ 169.6, 122.1, 120.6, 43.8, 31.2. For more information and clarifications, the Supplementary Material can be consulted (Figures S1 and S2).

The Fourier Transform Infrared Spectroscopy (FTIR) analysis contributed with the results from NMR. The compound showed absorption in the different regions and bands compatible with the formation of a cyclic amide (see the infrared spectrum of δ-lactam **1** in Figure S3 of Supplementary Material).

The melting point (MP) of the δ-lactam **1** resulted in MP = (105.2 ± 0.8) °C. Baron et al. (2011) determined the MP of the synthetic δ-lactam **1** and recorded values between 103 and 104 °C, very close to the MP attained in this work. Differences may be related to the purity of the compound isolated in this work or to the equipment accuracy.

δ-lactam **1** in caferana seeds extracts was quantified in extracts (Table 4) by calibration curve (concentration range from 0 to 50 ppm), which was constructed with a previously isolated and purified standard. Higher concentration of δ-lactam **1** was quantified in extract SC2, followed by SC1 extract, and PLE lastly. Considering that δ-lactam **1** is an alkaloid, it may have been influenced by conditions that also favored the extraction of caffeine in the SC2 extraction, as temperature and pressure that modified the solvent polarity. Once this is an unprecedented outcome, further investigations are necessary to determine more efficient parameters for obtaining δ-lactam **1** or enriched extracts.

Lactam compounds are described as a relatively new and distinct

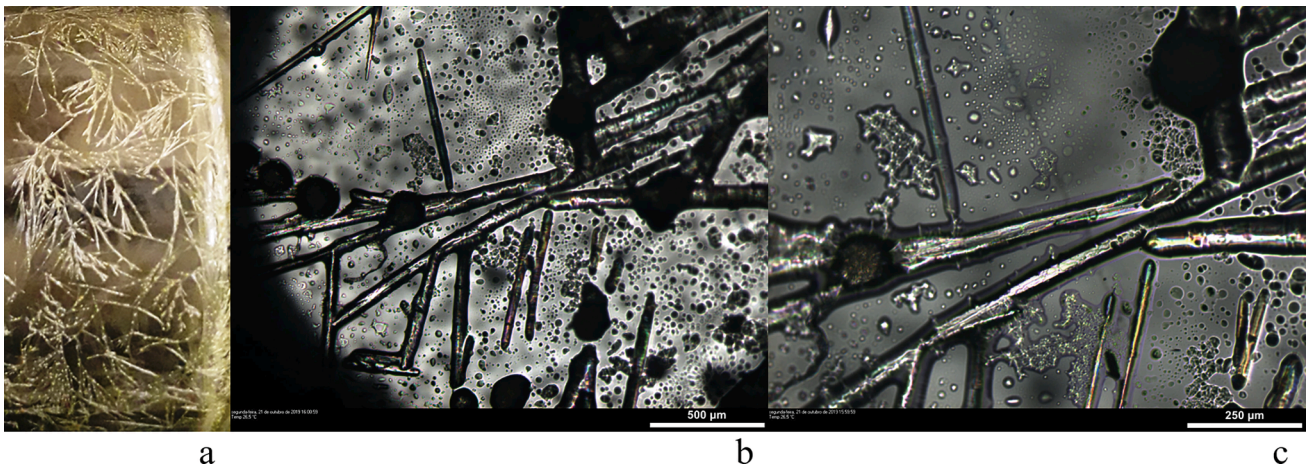


Fig. 1. (a) Structure-sized crystals in dried extract, (b) 5X zoom, (c) 10X zoom.

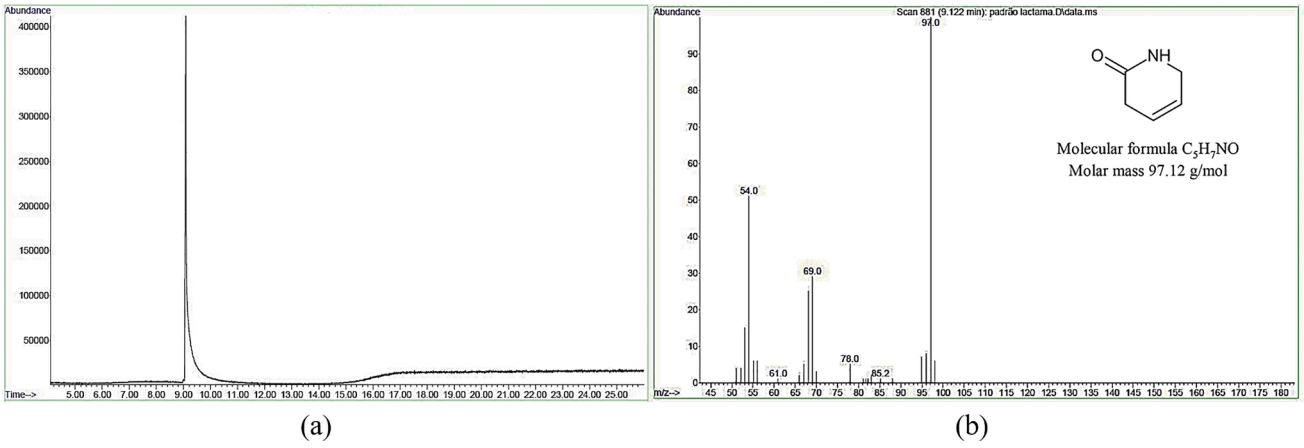


Fig. 2. (a) GC–MS chromatogram; (b) mass spectrum, structure, molecular formula, and molar mass of δ-lactam **1**.

class of natural products that may exhibit biological activities that vary from, antibacterial and antifungal to cytotoxic, phytotoxic, hypoallergenic, and anti-inflammatory. Lactam with 5 to 7 membered structures (γ-, δ-, σ-lactam) may have activities against degenerative diseases such as Parkinson's, Alzheimer's, anti-cancer, anti-viral, and anti-fungal agents (Albrecht et al., 2014; Assaf et al., 2014; Prando & Batistella, 2008). The probable action of these compounds in neurodegenerative diseases, along with the presence of maleimide, caffeine, and phenolics may indicate a way to elucidate the descriptions of physical and mental well-being reported by elderly people, who consume seeds and pulp from caferana fruits (Fraga et al., 2020; Peixoto et al., 2017).

Conclusions

This unprecedented study showed that caferana seeds have the potential to be used as an ingredient of products with a nutritional content or dietary supplement, as they contain high levels of protein, carbohydrates, soluble solids, and alkaloids, as caffeine and δ-lactam **1**. It contains 17 amino acids, 9 of which are essential to the human organism. They contain reducing sugars as glucose, fructose, maltose, arabinose, gal-xylose, and non-reducing sugars as sucrose, and 2 polyols, inositol, and sorbitol, being the last the main carbohydrate found in caferana seeds powder.

The volatile compounds showed a predominance of two functional groups, imine and aldehydes. The maleimide was found in high concentration in the volatile fraction. Although such molecule has no odor characteristic, it is a precursor of several nutraceutical compounds. The seed odor may be attributed to the aldehydes, mainly hexanal.

Extractions with supercritical CO_2 and ethanol as a co-solvent (SC1 and SC2) led to extracts with high caffeine and δ-lactam **1** contents, whereas higher total phenolic compounds amount was quantified in extracts from pressurized ethanol extraction (PLE).

The chemical composition of caferana seeds and its extracts evaluated in this study supports the popular knowledge that describes the physical and mental well-being of its consumers. It also brings the importance of adopting extraction protocols with renewable and GRAS solvents, aiming the obtainment of concentrated extracts free of contaminating residues, providing thus greater safety and health for consumers. Besides, environmental maintenance and added value to by-products from local production and familiar farming.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2021.100161.

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