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# Chemical profile of persian lime seeds (*Citrus Limettioides* T.): Focus on limonoids and polyphenols

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**Abstract:** Citrus fruit industrial processing generates tons of waste composed of peels, seeds and pulp. Incorrect disposal of these residues may harm the environment. The extraction of oil and bioactive compounds from citrus fruit seeds may be considered a sustainable alternative to the disposal of waste by the citrus agroindustry. In order to provide safe disposal of citrus waste an evaluation of its composition is necessary. Here we report the results of the application of a methodology to evaluate the composition the seeds of *Citrus limettioides*. In the first step, extraction with supercritical carbon dioxide was used. This work allowed the isolation and identification of four aglycone-type limonoids by High Performance Liquid Chromatography and Nuclear Magnetic Resonance, identified as limonin, nomilin, deacetylnomilin, and obacunone. In addition, six other polar limonoids and two glycosyl flavonoids were identified by HPLC-ESI/MS/MS.

**Key words:** *Citrus*, chemical profile, Persian lime, seeds, supercritical fluid extraction.

## INTRODUCTION

The global production of citrus fruits was about 158.5 million tons, in 2020. Asia has the highest production of these fruits (47%), followed by Africa (43.7%), America (8.1%), Europe (0.4%) and Oceania (0.1%). China leads citrus production with 28.16% of world production. Brazil, India and Mexico each account for about 5% of global production (Suri et al. 2022). The residues from the citrus agroindustry are rich sources of vitamins, mineral salts and dietary fiber, in addition to containing biologically active substances such as flavonoids, carotenoids, low molecular weight phenolics, terpenes, and considerable amounts of lipids, sugars, polysaccharides and organic acids. Some of these compounds have therapeutic properties such as: antioxidant, anticancer, antitumor, anti-inflammatory and antiviral, in addition to being a source of raw material of industrial interest (citrus pectin). For this reason, peels, bagasse and seeds can be considered important raw materials for the recovery of these bioactive compounds (Banerjee et al. 2017, Sharma et al. 2017). Part of the waste from this agroindustry supplies the production of animal feed or fertilizers, but the high cost of drying and transporting this material makes the cost of its processing a limiting factor for their use. Incorrect disposal of these residues may harm the environment. However, their high availability aroused the interest of researchers in adding value to waste from citrus fruit processing industries (Kobori & Jorge 2005).

Citrus fruit seeds are rich in vegetable oils with high nutritional value, proteins, limonoids, phytosterols, tocopherols and polyphenols (Kobori & Jorge 2005, Waheed et al. 2009). Limonoids are highly oxygenated

tetranortriterpenoids present in species of the Rutaceae and Meliaceae families. Seeds, fruits and peels of oranges (sweet and sour), lemons, limes, grapefruits, bergamots and mandarins are the main source of limonoids in *Citrus* genus. These compounds may be found both in the form of free aglycones as well as in the corresponding β-D-glycosides, being free aglycones mostly present in seeds and peels, while glycosides are formed during fruit maturation (Gualdani et al. 2016). Limonoids have antioxidant, anti-inflammatory, neuroprotective, antiviral, antimicrobial, antiprotozoal, antimalarial and antifungal activities, besides insecticidal activity. Figure 1 displays the structures of the main *Citrus* limonoids. Limonin is the major limonoid present in species of the genus *Citrus* (Zhang & Xu 2017, Hasegawa et al. 2000).

*Citrus* fruits belong to the genus *Citrus* of the Rutaceae family (Waheed et al. 2009). The genus *Citrus* is represented by several species with agroeconomic importance such as *Citrus sinensis* (common orange), *Citrus limon* (Sicilian lemon), *Citrus latifolia* (Tahiti acid lime), *Citrus grandis* (pomelo), *Citrus paradisi* (grapefruit), *Citrus medica* (cider), *Citrus reticulata* (mandarin, tangerine), and *Citrus aurantium* (bitter orange), being one of the most cultivated genera in the world (Araujo & Salibe 2002). The species *Citrus limettioides* Tanaka, popularly known as the Chick lime, sweet lime, navel lime or Persian lime originates from northern India and is commercially cultivated in several countries, mainly in warm and temperate tropical regions (Fronza & Hamann 2015). The fruit of this plant has a smooth peel, pale greenish-yellow in color and is very juicy (more than 50% by weight in juice) having an average of ten seeds (Lopes et al. 2013). Seeds have 20 – 37% of its weight as an oil (Waheed et al. 2009).

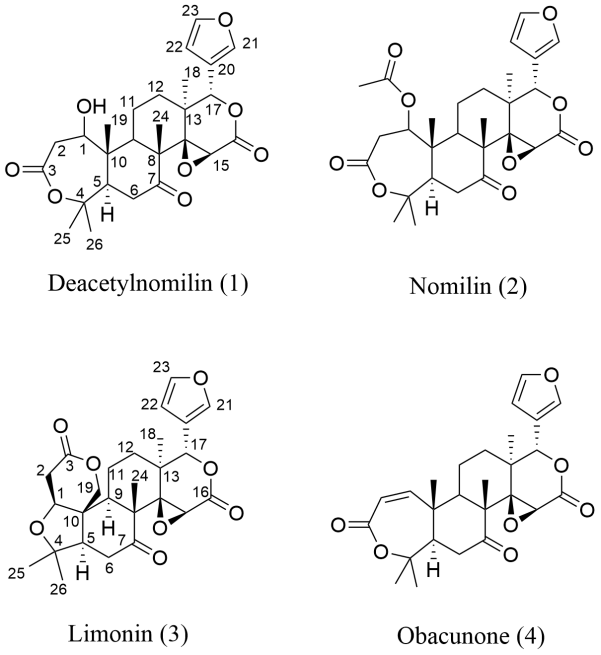


**Figure 1. Chemical structures of the main limonoids found in *Citrus limettioides*.**

Industrial strategies leading to more efficient extractions of bioactive substances promote the valorization of these products. In addition to avoiding degradation of thermolabile substances and the extensive use of solvents, they satisfy environmental regulations regarding waste disposal. In this context, extraction with supercritical fluid (SFE) appears as an alternative to conventional extraction methods. SFE is in line with Green Chemistry postulates and has the potential to provide high yields, reduce chemical degradation and overcome environmental constraints by reducing waste disposal (Putnik et al. 2017, Herrero et al. 2010).

In the present report, we present a separation scheme that allowed the extraction/characterization of the chemical composition of *C. limettioides* seeds, specifically, its oil, limonoids and polyphenols present therein. The methodology adopted allowed the characterization of the seed oil and the identification of sixteen substances, namely eight aglycones, six glycosylated limonoids, and two flavonoids. Our results contribute to the characterization of the phytochemical profile of

*Citrus limettioides* seeds, being the first report on the presence of glycosylated limonoids in this species.

## MATERIALS AND METHODS

### Standards and Reagents

Solvents used in extraction, HPLC and HPLC/MS were all of high purity. The carbon dioxide ($CO_2$) solvent used was of high purity (99.9%), from Air liquide (America Corp, Augusta, GA).

### Botanical material

Persian lime (*Citrus limettioides*) seeds were obtained from fruits purchased at CEASA commercial center, Rio de Janeiro. The seeds were dried in an oven at 40 °C and crushed in a laboratory blender (Waring Blender) before extraction.

### Preparation of Extracts

The supercritical fluid extractor used was a TOP INDUSTRIE (France) equipped with an XU032 oven and HPFlow PUMP 50-1000 pump. The crushed and dried material (26.6 g) was subjected to supercritical fluid extraction after packaging in a stainless steel extractor cell whose volume is 100 mL. The extraction parameters were those optimized by Yu *et al* for the extraction of limonoids from *C. paradisi* (Yu et al. 2007). Therefore, the operational conditions used were: 483 bar pressure, 50 °C temperature, supercritical $CO_2$ flow rate: 40 g/ min, extraction time: 60 minutes. A viscous, yellowish oil was obtained (extraction yield: 10.4 g, 39%).

The cake resulting from the supercritical $CO_2$ extraction (16 g) was placed in a Soxhlet extractor and was extracted with methanol (400 mL) for 6 hours. The resulting material was concentrated under vacuum on a rotary evaporator. 3.3 g (20%) of dry extract were obtained.

### Isolation and identification of limonoid aglycones

The crude extract obtained by supercritical $CO_2$ extraction was fractionated by partitioning between hexane, methanol and water (2:1:1, v/v/v), into three 200 mL portions. The hexane fraction obtained was concentrated under vacuum to obtain an oily material with a predominant composition of triglycerides. After alkaline hydrolysis and methylation with $BF_3$/ methanol (EDER 1995), the oil was submitted to gas chromatography coupled to mass spectrometry (Shimadzu QP 2010 Plus) under the following conditions: DB5-MS capillary fused silica column (30 m, 0.25 mm I.D., 0.25 µm film thickness). Quantitation was made on the basis of their chromatographic peak area percentages. The initial oven temperature was set at 50 °C (1min), then raised by 50 °C/min to 170 °C, 4 °C /min to 300 °C and then held for 20 min. He (99.999%) was used as the carrier gas with a flow rate of 1.0 mL/min; the injector temperature was set to 220 ºC and a split ratio of 1:50 was used. The oil was diluted in chloroform (5 µL oil in 450 µL chloroform) and 1 µL of the dilution was injected in the chromatograph. Mass spectra were taken at 70 eV. The mass range values used were of 40-700 Da.

The polar fraction (MeOH: $H_2O$) was concentrated *in vacuo* and subjected to one more partition using dichloromethane and water (2:1, v/v), in three 200 mL portions. The polar fraction in dichloromethane was submitted to semi preparative HPLC in a Shimadzu chromatograph composed of: DGU-20A degasser, LC-20AR pumps, SPD-20a UV/VIS detector, CBM-20A communicator and 100 µL loop, controlled by the LabSolutions program. The column used was Inertsyl ODS-4 RP18 (250 x 6.0 mm, 5 µm). semi-preparative mode, under the following chromatographic conditions: mobile phase (A): 0.1% aqueous formic acid and

(B): acetonitrile. The elution gradient used was: 0 min. = 46 % B, 30 min. = 100% B, 35 min. = 100% B, 40 min. = 46% B. The flow rate used was 1.7 mL/min. The separation took place at room temperature and the detection of the substances was performed with the aid of a UV absorption detector at 210 nm. About 20 mg of the fraction was dissolved in the initial mobile phase and filtered using a syringe filter (Nylon, 0.22 µm). HPLC fractionation resulted in the isolation of the limonoids deacetylnomilin (1,7 mg), nomilin (2 mg), limonin (5 mg) and obacunone (3 mg). NMR spectra of the isolated substances are in Supplementary Material - Figures S1, S2, S3, S4, S5, S6, S7, S8, S9.

**Deacetylnomilin (1):** $C_{26}H_{32}O_8$; $^1$H NMR (500 MHz, DMSO-d6), δ: 7.71 (s, H-23), 7.65 (s, H-21), 6.50 (s, H-22), 5.43 (s, H-17), 3.71 (s, H-15), 3.64 (t, H-1), 3.02 (t, H-2a), 2.67 (dd, H-9), 2.63 (t, H - 6b), 2.43 (dd, H- 5 ), 2.29 (dd, H-6a), 1.98 (s, H-18), 1.70 – 1.74 (m, H-12), 1.67 (m, H-11), 1.46 (s, H-26), 1.40 (m, H-11), 1.27 (s, H-25), 1.12 (s, H-19), 1.10 (s, H-24). 13C NMR (125 MHz, DMSO-d6), δ: 68.4 (C-1), 39.0 (C-2), 170.2 (C-3), 83.9 (C-4), 49.4 (C-5), 38.8 ( C-6), 208.6 (C-7), 52.2 (C-8), 43.7 (C-9), 44.5 (C-10), 16.9 (C-11), 31.2 (C-12), 36.9 (C -13), 65.8 (C-14), 53.0 (C-15), 167.2 (C-16), 77.6 (C-17), 20.9 (C-18), 16.0 (C-19), 120.1 (C-20), 143.4 (C-21), 110.0 (C-22), 141.5 (C-23), 16.0 (C-24), 32.9 (C-25), 23.1 (C-26) (Khalil et al. 2003).

**Nomilin (2):** $C_{28}H_{34}O_9$; $^1$H NMR (400 MHz, Chloroform-*d*), δ: 7.39 (s, H-21.23), 6.32 (t, *J* = 1.4 Hz, H-22), 5.44 (s, H-17), 5.01 (d , *J* = 7.1 Hz, H-1), 3.79 (s, H-15), 3.20 (dd, *J* = 15.6, 7.1 Hz, H-2a), 3.10 (dd, *J* = 15.6, 7.1 Hz, H-2b ), 2.76 (t, *J* = 14.9 Hz, H-6), 2.58 (dd, *J* = 14.7, 3.6 Hz, H-5, 6), 2.47 (dd, *J* = 9.7, 3.3 Hz, H-9), 2.01 (s, H(OAc)), 1.78 (m, H-11), 1.61 (m, H-12), 1.55 (s, H-26), 1.46 (s, 25), 1.32 (s, H- 19), 1.13 (s, H-24), 1.10 (s, H-18). $^{13}$C NMR (100 MHz, Chloroform-*d*), δ: 70.68 (C-1), 35.15 (C-2), 169.00 (C-3), 84.3 (C-4), 51.0 (C-5), 38.8 ( C-6), 206.6 (C-7), 52.8 (C-8), 44.3 (C-9), 44.0 (C-10), 16.6 (C-11), 32.2 (C-12), 37.4 (C -13), 65.4 (C-14), 53.3 (C-15), 166.6 (C-16), 78.0 (C-17), 17.1 (C-18), 17.1 (C-19), 120.11 (C- 20), 143.2 (C-21), 109.6 (C-22), 141.0 (C-23), 20.6 (C-24), 33.4 (C-25), 23.3 (C-26), 169.2 (OAc), 20.6 (OAc-Me) (Manners et al. 2000).

**Limonin (3):** $C_{26}H_{30}O_8$; $^1$H NMR (500 MHz, chloroform-*d*), δ: 7.40 (d, H-21, 23), 6.34 (d, *J* = 1.9 Hz, H-22), 5.47 (s, H-17), 4.77 (d , *J* = 13.1 Hz, H-19a), 4.47 (d, *J* = 13.1 Hz, H-19b), 4.04 (s, H-15), 2.98 (dd, *J* = 16.8, 3.8 Hz, H-2b), 2.86 (t, *J* = 15.2 Hz, H-6b), 2.68 (d, J = 16.8 Hz, H-2a), 2.55 (dd, J = 12.4, 2.9 Hz, H-9), 2.47 (dd, H- 6a), 2.23 (dd, *J* = 15.9, 3.3 Hz, H-5), 1.71 – 1.90 (m, H-11), 1.51 (m, H-12), 1.30 (s, H-25), 1.18 ( d, *J* = 2.8 Hz, H-18, 26), 1.07 (s, H-24). $^{13}$C NMR (125 MHz, chloroform-*d*), δ: 79.15 (C-1), 35.65 (C-2), 169.17 (C-3), 80.34 (C-4), 60.55 (C-5), 36.39 ( C-6), 206.10 (C-7), 51.34 (C-8), 48.13 (C-9), 45.94 (C-10), 18.92 (C-11), 30.86 (C-12), 37.96 (C-11) -13), 65.67 (C-14), 53.85 (C-15), 166.64 (C-16), 77.81 (C-17), 20.72 (C-18), 65.36 (C-19), 119.97 (C- 20), 143.24 (C-21), 109.67 (C-22), 141.12 (C-23), 17.61 (C-24), 30.16 (C-25), 21.38 (C-26) (Poulose et al. 2007).

**Obacunone (4):** $C_{26}H_{30}O_7$; $^1$H NMR (500 MHz, chloroform-*d*), δ: 7.41 (d, H-21, 23), 6.51 (d, *J* = 11.7 Hz, H-1), 6.37 (s, H-22), 5.97 (d , *J* = 11.7 Hz, H-2), 5.46 (s, H-17), 3.65 (s, H-15), 2.97 (t, *J* = 14.1 Hz, H-6b), 2.60 (dd, *J* = 14.1 , 5.0 Hz, H-5), 2.30 (dd, H-6a), 2.15 (m, H-9), 1.90 – 1.80 (m, H-11, 12b), 1.51 (s, H-19, 26) , 1.45 (m, H-12), 1.46 (s, H-25), 1.25 (s, H-24), 1.13 (s, H-18). $^{13}$C NMR (125 MHz, chloroform-*d*), δ: 156.74 (C-1), 123.03 (C-2), 166.9 (C-3), 83.96 (C-4), 57.36 (C-5), 39.92 ( C-6), 207.39 (C-7), 53.36 (C-8), 49.24 (C-9), 43.15 (C-10), 19.48 (C-11), 32.80 (C-12), 37.45 (C -13), 65.04 (C-14), 52.96 (C-15), 166.6 (C-16), 77.89 (C-17), 21.15 (C-18), 16.45 (C-19), 120.10 (C- 20), 141.03 (C-21), 109.67 (C-22), 143.19 (C-23), 17.00 (C-24), 26.81 (C-25), 32.05 (C-26) (Poulose et al. 2007).

## Nuclear Magnetic Resonance

$^1$H/$^{13}$C NMR spectra were obtained at 500/125 MHz and 400/100 MHz (Varian VNMRSYS-500), in the indicated solvents, with TMS as internal standard, using 3 mm tubes. The 2D correlation

experiments were performed with the help of proprietary software. For 2D heteronuclear experiments (edited HSQC, HMBC), typical acquisition parameters included 1K or 2K x 512 data points, filled with zeros to 1K or 2K x 2K points, and processed using linear prediction in F1. All spectral data were processed using MestReNova software version 12.0.

### Characterization of the composition of the polar fraction

The methanolic extract obtained from the cake in the Soxhlet extractor was partitioned between dichloromethane and water (2:1, v/v), with volumes of 200 mL each, three times. The aqueous fraction resulting from this partition was analyzed by HPLC/DAD in analytical mode and by mass spectrometry (HPLC-MS) with electrospray interface (ESI) in negative mode (see conditions below). For both methods, the solvent system used was: (A) 0.1% aqueous tetrahydrofuran and (B) acetonitrile. Separation was performed in gradient mode, ranging from 100% A to 100% B for 60 minutes. The column used in these analyzes was a Waters Symmetry RP18 (150 mm x 4.6 mm, 5 µm) and the flow rate was 0.4 mL/min. This fractionation resulted in the identification of 6 glycosylated limonoids: nomilin 17-β-D-glucopyranoside (NG) (5), limonin 17-β-D-glucopyranoside (LG) (6), obacunone 17-β-D-glucopyranoside (OG) (7), nomilinic acid 17-β-D-glucopyranoside (NAG) (8), deacetylnomilinic acid 17-β-D-glucopyranoside (DNAG) (9) and deacetylnomilinic 17-β-D-glucopyranoside (DNG) (10), in addition to four aglycone-type limonoids, limonoic acid (11), deacetylnomilinic acid (12), isolimonoic acid (13) and nomilinic acid (14), and two flavonoids: vicenin-2 (apigenin 6,8-di-C-glucoside) (15) and brutieridin (16) (Figure 2). The identification criteria were based on MS/MS data and UV absorption spectra.

## HPLC-UV-MS/MS

The analyzes by high performance liquid chromatography with ultraviolet detector coupled to tandem mass spectrometry (HPLC-UV-MS/MS) were performed using a Dionex UltiMate 3000 system, coupled to a Fleet LCQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA. A Waters Symmetry RP-18 column (150 x 4.6 mm, 5 µm) was used at a flow rate of 0.4 mL $min^{-1}$. Separation was performed at room temperature and the mobile phase used was: 0.1% aqueous trifluoroacetic acid (A) and acetonitrile (B). The gradient used was: 100% A to 100% B in 65 minutes. The mass spectrometer, equipped with an electrospray source (ESI), was operated in negative ionization mode. High purity nitrogen was used as sheath gas (35 arbitrary units) and auxiliary gas (10 arbitrary units). High purity helium was used as collision gas. The instrumental parameters used were as follows: source voltage: 5 kV, capillary voltage: 7 V, tube lens: 65 V and capillary temperature: 400 °C. Mass spectra were acquired in the range of 140–1500 Da. For the fragmentation study, a data-dependent scan was performed and the normalized collision energy of the collision-induced dissociation (CID) cell was set to 30 eV and the isolation width of precursor ions was set to m/z 2.0.

## RESULTS AND DISCUSSION

An extraction scheme for the phytochemical study of *C. limettioides* seeds was developed and its application enabled the extraction/characterization of the chemical composition of fruit seeds, specifically, their oil, limonoids and polyphenols present therein. In the first phase of the scheme, application of supercritical fluid extraction with supercritical $CO_2$ yielded 39% of oil composed of triglycerides of palmitic (26.7%),

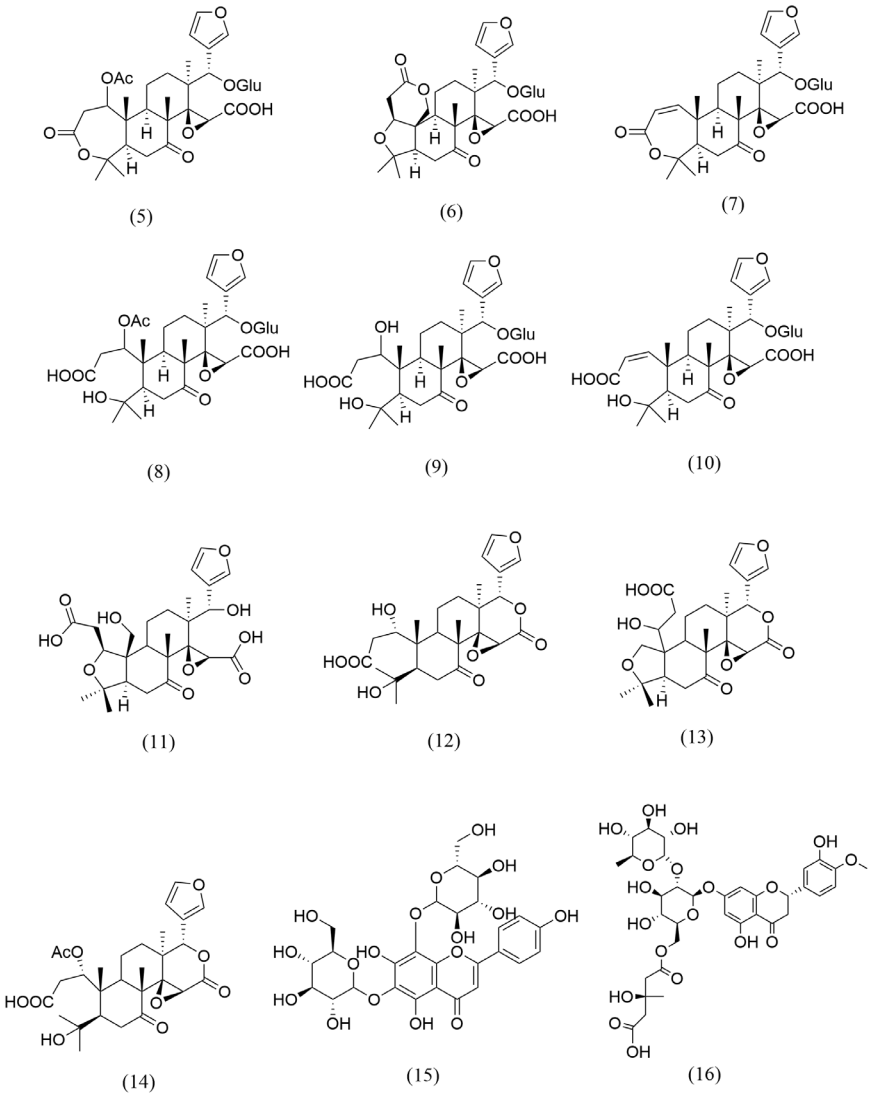


**Figure 2. Structures of compounds identified in the aqueous fraction of *C. limettioides*.**

stearic (3%), oleic (30.3%), and linoleic acids (29.9%), and other minor fatty acids, besides other compounds. Partition of the oil between water, methanol and chloroform and preparative HPLC led to the isolation and identification of four limonoids, namely: deacetylnomilin (1), nomilin (2), limonin (3) and obacunone (4). The structures of the isolated substances were determined by comparison of their spectroscopic data obtained by $^{1}H$ and $^{13}C$ NMR, with data from the literature (Manners et al. 2000, Khalil et al. 2003, Poulose et al. 2007). Rouseff and Nagy (1982), using gas chromatography, studied the content and identity of limonoids in seeds of several Citrus species, including *C. limettioides*, having identified the same limonoids as above, noting that limonin is the major limonoid in this and the other studied species.

In the aqueous fraction obtained from the cake resulting from the $CO_2$ extraction, eleven substances were characterized by HPLC-UV/DAD/ESI-MS/MS analysis in the negative mode, as shown in Table I. The mass spectra of the fraction produced abundant deprotonated molecular ions by ESI in negative mode, with little fragmentation in the analysis under conditions of Collision Induced Dissociation (Energy of 30 eV). The compound identification criteria was

**Table I.** Characterization of the substances present in the aqueous fraction of *C. limettioides.*

| Elution order | *Rt* (min) | UV max. (nm) | $[M-H]^-$ | Nominal mass | MS/MS | Molecular formula | Proposed compound |
|---|---|---|---|---|---|---|---|
| 1 | 18.84 | 271/334 | 593.3 | 594.5 | 503.1/ 383.2 | $C_{27}H_{30}O_{15}$ | Apigenin 6,8-di-C-glucoside (**15**) |
| 2 | 20.08 | 275/346 | 753.1 | 738.7 | 651.0/591.0 | $C_{34}H_{42}O_{18}$ | Brutieridin (**16**) |
| 3 | 24.99 | 210 | 649.2 | 650.6 | 605.2/ 443.0 | $C_{32}H_{42}O_{14}$ | Limonin 17-β-D-glucopyranoside (LG) (**6**) |
| 4 | 25.28 | 210 | 669.3 | 670.7 | 609.3 | $C_{32}H_{46}O_{15}$ | Deacetylnomilinic acid 17-β-D-glucopyranoside (DNAG) (**9**) |
| 5 | 26.11 | 210 | 651.4 | 652.2 | 565.1/ 345.0 | $C_{32}H_{44}O_{14}$ | Deacetylnomilinic 17-β-D-glucopyranoside (DNG) (**10**) |
| 6 | 28.40 | 210 | 693.4 | 694.7 | 565.0/ 427.2 | $C_{34}H_{46}O_{15}$ | Nomilin 17-β-D- glucopyranoside (NG) (**5**) |
| 7 | 28.88 | 210 | 711.3 | 712.7 | 607.1/ 651.0 | $C_{34}H_{48}O_{16}$ | Nomilinic acid 17-β-D-glucopyranoside (NAG) (**8**) |
| 8 | 29.31 | 210 | 633.4 | 634.6 | 427.1 | $C_{32}H_{42}O_{13}$ | Obacunone 17-β-D- glucopyranoside (OG) (**7**) |
| 9 | 30.99 | 209 | 505.2 | 506.5 | 487.1/415.0 | $C_{26}H_{30}O_{10}$ | Limonoic acid (**11**) |
| 10 | 32.93 | 209 | 489.2 | 490.5 | 471.1/ 333.0 | $C_{26}H_{34}O_{9}$ | Deacetylnomilinic acid (12) |
| 11 | 33.79 | 209 | 487.2 | 488.5 | 383.1/347.1 | $C_{26}H_{32}O_{9}$ | Isolimonoic acid or limonoic acid A-ring lactone (13) |
| 12 | 36.02 | 209 | 531.0 | 532.6 | 471.0/ 427.0 | $C_{28}H_{36}O_{10}$ | Nomilinic acid (**14**) |

based on the ultraviolet absorption spectra and their MS/MS spectra, with data obtained in MS1 (mass of the deprotonated molecular ion) and by the fragmentation pattern (in MS2).

Mass spectrometry of limonoids has been an important tool in studies on the structure of limonoids. Tian et al. (2003) studied the fragmentation of citrus limonoids after electroionization. Still in 2003, Tian and Schwartz carried out a very comprehensive study of the fragmentation of aglycones and limonoid glycosides, this time with electrospray ionization and atmospheric pressure ionization, in negative and positive modes using collision-induced dissociation and sequential mass spectrometry (Tian & Ding 2000). Avula et al. (2016) studied limonoids and flavonoids in citrus fruit seeds.

Fragmentation of limonoid glycosides is dominated by the elimination of the glucosyl moiety (Jayaprakasha et al. 2010). In the mass spectrum of obacunone 17-β-D-glucopyranoside, OG (7) and nomilin 17-β-D-glucopyranoside, NG (5) intense deprotonated ions $[M-H]^-$ at m/z 633.4 and m/z 693.4, respectively, were observed. The fragmentation of the ion at m/z 633.4 resulted in an intense ion at m/z 427.1, which corresponds to loss of a neutral fragment [$CO_2$+glucose] for OG (Tian & Ding 2000). The fragment at m/z 565.1 in the NG mass spectrum was already noticed by Tian & Ding (2000) and by Raman et al. (2005), but no proposal for the fragmentation was suggested by these authors.

In the limonin 17-β-D-glucopyranoside, LG (6), ($[M-H]^-$ = 649.2), MS/MS spectrum, the fragment at m/z 605.2 may be attributed to the loss $CO_2$ from the deprotonated molecular ion. An additional glucosyl neutral loss leads to m/z 443 ion (Jayaprakasha et al. 2011).

Similar to the spectrum found in the literature for nomilinic acid 17-β-D-glucopyranoside, NAG

([M-H]$^-$ = 711.3), (8) two fragments were observed at m/z 607.1 ([M-H]$^-$ - $CO_2$ - $CH_3COOH$) and m/z 651 ([M-H]$^-$ - $CH_3COOH$). Not many fragmentations were observed in the deacetylnomilic acid 17-β-D-glucopyranoside, DNAG (9) and deacetilnomilinic 17-β-D-glucopyranoside, DNG (10) spectra. The MS/MS spectrum of DNAG ([M-H]$^-$ = m/z 669.3) showed only one intense peak at m/z 609.3 (([M-H]$^-$ - $CH_3COOH$). The DNG (([M-H]$^-$ = 651.7) MS/MS spectrum showed an intense ion at m/z 489.2, which corresponds to a probable loss of [H + glucose] (Avula et al. 2016).

The acid limonoids **11**, **12, 13** and **14** exhibited deprotonated molecular ions at m/z 505.2, 489.2, m/z 487.2 and m/z 531.0, respectively. These substances produced fragments due to the neutral loss of $H_2O$ and acetic acid in the MS-MS spectra (Avula et al. 2016, Guccione et al. 2016, Sommella et al. 2014, Liu et al. 2021).

The spectrum of apigenin 6,8-di-C-glycoside (**15**) displays a deprotonated ion at m/z 593.3. The MS/MS spectrum showed an intense ion at 353 [(M – H)-240)]$^-$, in addition to fragments at 383.08 [(M – H)-210)]$^-$, 473 [(M – H)- 120)]$^-$, 503 [(M – H)-90]$^-$ and 575 [(M – H)-18]$^-$, as described in the literature (Guccione et al. 2016, Sommella et al. 2014). The flavonoid brutieridin (**16**) exhibited an m/z ion of 753.1 [(M-H)]$^-$ . The MS/MS spectrum of the deprotonated ion showed fragments at m/z 591.0, corresponding to the loss of a glucosyl moiety, in addition to fragments at m/z 609 and m/z 651.22 (Sommella et al. 2014, Guccione et al. 2016).

Limonoids are widely distributed in a wide variety of species of the genus *Citrus*. However, reports dealing specifically with this class of compounds are scarce. In a work carried out by Rouseff & Nagy (1982) limonoids in seeds of *C. limettioides* T. were determined by HPLC by comparing the retention time of limonoids with standards. The following limonoids were identified: limonin, obacunone, nomilin, deacetylnomilin and deoxylimonine. However, the presence of glycosylated limonoids was not reported.

## CONCLUSION

An extraction scheme was designed and applied to the phytochemical study of *C. limettioides* seeds. In the first step, the seeds were extracted with supercritical $CO_2$. The resulting solid residue was then submitted to liquid-liquid partitions. The application of this extraction scheme resulted in the isolation of four aglycone-type limonoids (deacetylnomilin, nomilin, limonin and obacunone) from the extract obtained with $CO_2$. The subsequent liquid-liquid partition steps led to a fraction rich in polar components, whose composition was elucidated with the aid of HPLC-MS/MS. Four aglycone-type acidic limonoids (limonoic acid, deacetylnomilinic acid, isolimonoic acid or limonoic acid A-ring lactone and nomilinic acid), six glycosylated limonoids were identified: nomilin 17-β-D-glucopyranoside, limonin 17-β-D-glucopyranoside, obacunone 17-β-D-glucopyranoside, nomilinic acid 17-β-D-glucopyranoside, deacetylnomilinic acid 17-β-D-glucopyranoside and deacetylnomilinic 17-β-D-glucopyranoside, in addition to two flavonoids: vicenin-2 (apigenin 6,8-di- C-glycoside) and brutieridin. This is the first report describing the presence of glycosylated limonoids in *Citrus limettioides* T. seeds. The review by Gualdani et al. (2016) pointed out that the reason for the low number of studies on the biological activities of these compounds may be attributed to their difficult isolation from natural sources and to their poor pharmacokinetics. Thus, the separation/analysis scheme reported here can be considered a general scheme for the analysis and separation of metabolites in citrus seeds in general.

## REFERENCES


ARAUJO JRG & SALIBE AA. 2002. Caracterização físico-morfológica de frutos de microtangerinas (*Citrus* spp.) de potencial utilização como porta-enxertos. Rev Bras Frutic 24: 618-621.

AVULA B, SAGI S, WANG YH, WANG M, GAFNER S, MANTHEY J & KHAN I. 2016. Liquid Chromatography-Electrospray Ionization Mass Spectrometry Analysis of Limonoids and Flavonoids in Seeds of Grapefruits, Other Citrus Species, and Dietary Supplements. Planta Med 82: 1058-1069.

BANERJEE J, SINGH R, VIJAYARAGHAVAN R, MACFARLANE D, PATTI AF & ARORA A. 2017. Bioactives from fruit processing wastes: Green approaches to valuable chemicals. Food Chem 225: 10-22.

EDER K. 1995. Gas chromatographic analysis of fatty acid methyl esters. Journal of Chromatography B 671: 113-131.

FRONZA D & HAMANN JJ. 2015. Em Frutíferas de clima tropical e subtropical, Rede e-Tec Brasil, Santa Maria: Universidade Federal de Santa Maria, p. 75.

GUALDANI R, CAVALLUZZI M, LENTINI G & HABTEMARIAM S. 2016. The Chemistry and Pharmacology of Citrus Limonoids. Molecules 21: 1530.

GUCCIONE C, BERGONZI M, PIAZZINI V & BILIA A. 2016. A Simple and Rapid HPLC-PDA MS Method for the Profiling of Citrus Peels and Traditional Italian Liquors. Planta Med 82: 1039-1045.

HASEGAWA S, BERHOW MA & MANNERS GD. 2000. Citrus Limonoid Research: An Overview. In: Citrus Limonoids, BERHOW M ET AL (Eds), ACS Symposium Series, American Chemical Society: Washington, DC.

HERRERO M, MENDIOLA JA, CIFUENTES A & IBÁÑEZ E. 2010. Supercritical fluid extraction: Recent advances and applications. J Chromatogr A 1217: 2495-2511.

JAYAPRAKASHA GK, DANDEKAR DV, TICHY SE & PATIL BS. 2011. Simultaneous separation and identification of limonoids from citrus using liquid chromatography-collision-induced dissociation mass spectra. J Sep Sci 34: 2-10.

JAYAPRAKASHA GK, JADEGOUD Y, NAGANA GOWDA GA & PATIL B S. 2010. Bioactive Compounds from Sour Orange Inhibit Colon Cancer Cell Proliferation and Induce Cell Cycle Arrest. J Agric Food Chem 58: 180-186.

KHALIL AT, MAATOOQ GT & EL SAYED KA. 2003. Limonoids from *Citrus reticulate*. Z Naturforsch C 58: 165-170.

KOBORI CN & JORGE N. 2005. Caracterização dos óleos de algumas sementes de frutas como aproveitamento de resíduos industriais. Cienc Agrotec 29: 1008-1014.

LIU Y, ZHAO F, ZHANG Z, LI T, ZHANG H, XU J, YE J & DENG X. 2021. Structural diversity and distribution of limonoids in pummelo (*Citrus grandis*) fruit revealed by comprehensive UHPLC-MS/MS analysis. Sci Hortic 282: 109996.

LOPES LTA, DE PAULA JR, TRESVENZOL LMF, BARA MTF, DE SÁ S, FERRI PH & FIUZA TS. 2013. Composição química e atividade antimicrobiana do óleo essencial e anatomia foliar e caulinar de *Citrus limettioide*s Tanaka (Rutaceae). Rev Ciênc Farm Básica Apl 34: 503.

MANNERS GD, HASEGAWA S, BENNETT RD & WONG RY. 2000. LC-MS and NMR Techniques for the Analysis and Characterization of Citrus Limonoids. In Citrus Limonoids: BERHOW M ET AL. (Eds), ACS Symposium Series, American Chemical Society: Washington, DC, Volume 758, Chapter 4, p. 40-59.

POULOSE SM, JAYAPRAKASHA GK, MAYER RT, GIRENNAVAR B & PATIL BS. 2007. Purification of citrus limonoids and their differential inhibitory effects on human cytochrome P450 enzymes. J Sci Food Agric 87: 1699-1709.

PUTNIK P, KOVAČEVIĆ BK, JAMBRAK AR, BARBA FJ, CRAVOTTO G, BINELLO A, LORENZO JM & SHPIGELMAN A. 2017. Innovative "Green" and Novel Strategies for the Extraction of Bioactive Added Value Compounds from Citrus Wastes—A Review. Molecules 22: 680.

RAMAN G, CHO M, BRODBELT JS & PATIL BS. 2005. Isolation and purification of closely related Citrus limonoid glucosides by flash chromatography. Phytochem Anal 16: 155-160.

ROUSEFF RL & NAGY S. 1982. Distribution of limonoids in Citrus seeds. Phytochemistry 21: 85-90.

SHARMA K, MAHATO N, CHO M H & LEE YR. 2017. Converting citrus wastes into value-added products: Economic and environmently friendly approaches. Nutr 34: 29-46.

SOMMELLA E, PEPE G, PAGANO F, TENORE GC, MARZOCCO S, MANFRA M, CALABRESE G, AQUINO RP & CAMPIGLIA P. 2014. UHPLC profiling and effects on LPS-stimulated J774A.1 macrophages of flavonoids from bergamot (*Citrus bergamia*) juice, an underestimated waste product with high anti-inflammatory potential. J Funct Foods 7: 641-649.

SURI S, SINGH A & NEMA PK. 2022. Current applications of citrus fruit processing waste: A scientific outlook. Appl Food Biotechnol 2: 100050.

TIAN Q & DING X. 2000. Screening for limonoid glucosides in *Citrus tangerina* (Tanaka) Tseng by high-performance liquid chromatography–electrospray ionization mass spectrometry. J Chromatogr A 874: 13-19.

TIAN Q, LI D, BARBACCI D, SCHWARTZ SJ & PATIL BS. 2003. Electron ionization mass spectrometry of citrus limonoids. Rapid Commun Mass Spectrom 17: 2517-2522.

TIAN Q & SCHWARTZ SJ. 2003. Mass Spectrometry and Tandem Mass Spectrometry of Citrus Limonoids. Anal Chem 75: 5451-5460.

WAHEED A, MAHMUD S, SALEEM M & AHMAD T. 2009. Fatty acid composition of neutral lipid: Classes of Citrus seed oil. J Saudi Chem Soc 13: 269-272.

YU J, DANDEKAR D, TOLEDO R, SINGH R & PATIL B. 2007. Supercritical fluid extraction of limonoids and naringin from grapefruit (*Citrus paradisi* Macf.) seeds. Food Chem 105: 1026-1031.

ZHANG Y & XU H. 2017. Recent progress in the chemistry and biology of limonoids. RSC Adv 7: 35191-35220.

## SUPPLEMENTARY MATERIAL

**Figures S1, S2, S3, S4, S5, S6, S7, S8, S9.**

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## Author contributions

Tairini Roberto da Silva carried out the experiments, interpreted the data and wrote the initial manuscript. Antonio Jorge R. da Siva designed the research, analysed the data, wrote and revised the final article.