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Original Article

Identification and quantification of limonoid aglycones content of *Citrus* seeds

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ABSTRACT

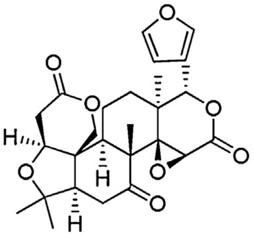
Citrus fruits are recognized as an important source of bioactive molecules such as limonin and nomilin. However, these molecules exhibit low bioavailability, therefore, obtaining these molecules using biotechnological techniques may be an alternative to harvesting them directly from fruits. The aim of this study was to quantify and identify limonoids in the dichloromethane extracts of *Citrus* seeds of Criolla orange, Oneco tangerine, Tangerine-lemon, Sour orange and Valencia orange from department of Antioquia-Colombia by high performance liquid chromatography with diode array detection, and high-resolution mass spectrometry. Although in all the samples total glycosidic free limonoids were present, Oneco tangerine seeds had the highest concentration, followed by Tangerine-lemon seeds, equivalent to 0.75% and 0.53% per total dry weight, respectively. These results suggest Oneco tangerine seeds may be used as an elite material for biotechnological processes looking for increased production of limonoids to support research and drug development.

Introduction

Limonoids are highly oxygenated triterpenoids widely distributed in the Rutaceae and Meliaceae families (Dreyer et al., 1972; Hasegawa et al., 1980; Coy Barrera et al., 2013) of the *Citrus* genus. Limonoids are classified as aglycones (LA) or glycosides (LG) (Jayaprakasha et al., 2011), and they contain a furan ring attached to ring D at C-17, as well as oxygen-containing functional groups at C-3, C-4, C-7, C-16, and C-17 (Roy and Saraf, 2006). Currently, 39 LA and 21 LG have been identified (Ohta et al., 1992; Patil et al., 2010).

Various biological activities of *Citrus*-derived limonoids have been reported *in vitro* and *in vivo*, including mosquito repellent, anti-inflammatory, anti-viral, anti-allergic, anti-proliferative, anti-mutagenic, and anti-carcinogenic (Jayaprakasha et al., 2011). The anti-*Leishmania* activity of LA and LG of the genus *Citrus* sp., *Raputia heptaphylla* and *Esenbeckia litoralis*, Rutaceae, has also been reported (Granados-Falla et al., 2016; Marin et al., 2016; Garcia et al., 2017). Although limonoids are bioactive, they are high-cost products, and are structurally complex, which hinders their synthesis. As such, biotechnological research, such as development of tissue culture techniques to improve limonoid production, is necessary (Roy and Saraf, 2006) to provide enough of these molecules to support research aimed at improving their bioavailability.

Limonoids are the most abundant terpenoids in *Citrus* fruits (Hasegawa et al., 1996), with limonin (**1**), nomilin (**2**), ubacunone, deacetyl nomilin, and ichangine present in high concentrations (Rouseff and Nagy, 1982; Ozaki et al., 1991). In *Citrus* sp., diacetyl nomilinic acid is the precursor for all limonoids, and is synthesized in the phloem of the stem from acetate and mevalonate pathway, through farnesyl pyrophosphate, and then translocated to the leaves, fruits, and seeds, where it is metabolized to other limonoids (Hasegawa et al., 1996; Roy and Saraf, 2006) resulting in tissue-specific production.



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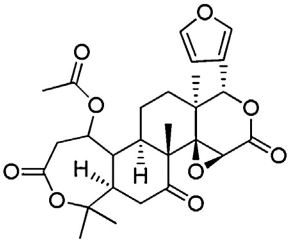

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Limonoids can be synthesized in seeds in both their free or glycosidic form (Tian et al., 2007); while LA accumulate during development of the tissue, LG accumulate during maturation of the

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seed (Ozaki et al., 1991). Studies on the distribution of limonoids in grapefruits have shown that seeds contain the highest limonoid content, followed by the membrane of the segments, and the leaves have the lowest content (Wang et al., 2016). The proportion of LG to LA in seeds is 1:2.2, making seeds an excellent source of LA (Wang et al., 2016) where limonin (**1**) and nomilin (**2**) are the most abundant components (Codoñer-Franch and Valls-Belles, 2010).

The objective of this work was to identify and quantify LA in dichloromethane extracts of *Citrus* seeds obtained from the department of Antioquia, Colombia to identify an elite material with high concentrations of LA as a starting point to evaluate their production by biotechnological processes.

Materials and methods

Plant material

Citrus fruits of Criolla orange (A-C): *Citrus* x *aurantium* L. (Sweet Orange group), Oneco tangerine (A-O): *Citrus reticulata* Blanco (A-O), Tangerine-lemon (A-L): *Citrus* cf. x *limonia* (L.) Osbeck and Sour Orange (A-A): *Citrus* x *aurantium* (Sour Orange group), each one approximately 8 years of age, were collected in the jurisdiction of the municipality of Andes, La Solita village, San Juan river basin, Colombia. Oneco tangerine (T-O): *Citrus reticulata* Blanco, Valencia orange (T-V): *Citrus* x *aurantium* (Sweet Orange group) of approximately 12 years old, were harvested in the municipality of Támesis, Pescadero village, Cartama river basin, Colombia and Oneco tangerine (B-O): *Citrus reticulata* Blanco and Valencia orange (B-V): *Citrus* x *aurantium* (Sweet Orange group) about 22 years old, were collected at the north of the Metropolitan Area of the Aburrá Valley, municipality of Barbosa, Yarumito village, located in the basin of the Porce River (Medellín), Colombia. Cultivation of *Citrus* fruits in Pescadero is commercial, while crops in La Solita and Yarumito are for internal consumption. The coordinates of the collection sites are shown in Figure S1 and Table S2.

Mature hesperidia were taken from different locations on the trees, the seeds of the fruits were extracted, and pooled for extraction of limonoids. The *Citrus* trees were georeferenced using a GPS (Garmin, GPSMAP 60CSx). Soil samples were taken from a depth of 30 cm using a driller at each sampling site, and samples were sent to the soil laboratory of the National University, Medellín for analysis. The botanical samples were determined in the herbarium of University of Antioquia and deposited with the codes: HUA 220323 to HUA 220330.

Extraction of limonoids

The seeds were washed, dried at 40 °C for 72 h, ground and stored at room temperature. The seeds were degreased with petroleum ether a rate of 17 ml/g, assisted by ultrasound for 20 min (37 kHz, 40 W, 40 °C). After 15 min, the supernatant was discarded and neutral limonoids were extracted by ultrasound with dichloromethane at 35 °C. The extracts were filtered and rotoevaporated at 35 °C and 120 rpm (Wang et al., 2016). All procedures were done in triplicate.

Quantification of limonoids by HPLC-DAD and High resolution mass spectrometry (HR-MS)

HPLC-HR-MS analyses were performed using a C18 column (Chromolith HighResolution, RP-18e 50–4.6 mm), with the solvents A = 0.1% formic acid in acetonitrile and B = 0.1% formic acid in water. The flow rate was 0.5 ml/min, and the injection volume was 10 μl. The conditions for the gradient program were: 7 min, 100% B; 7–9 min, 0–80% A; 9–11 min, 99% A; 11–13 min, 99% A; 13–14 min, 100% B. Column and autosampler temperature were 40 °C and 4 °C, respectively. The Bruker Impact II UHR-QqTOF (Bruker Daltonik), equipped with an ESI + source at 200 °C. Dry nitrogen was used as cone and desolvation gases at 8 l/min. The capillary voltage was set to 4500 V, and the collision energy was 5 eV. Data were collected scanning values ranged from *m/z* 50 to *m/z* 1200 and processed using Data Analysis 4.3.

The mass measurement error (accuracy) was calculated with the formula: Accuracy = ((Experimental mass-theoretical mass) × theoretical mass^{-1} × 1,000,000) ppm (Brenton and Godfrey, 2010). The required values are given in table S5.

Statistical analysis

Data were analyzed using ANOVA and post hoc Tukey's HSD test. Values of $p < 0.05$ were considered statistically significant. Estimates of total limonoids were determined from triplicate experiments, and the values are expressed as mean ± SD.

Results

Plant material

The *Citrus* fruits harvested in this study were located between 755 and 1279 m above sea level, in sandy loam soils (FAr) and sandy soils (A), with a percentage of silt between 14% and 30% (table S1). The pH values varied from 4.9 to 5.4 dsm-1, and the ratio of (Ca + Mg + K)/Al, ranged from 5.91 (Andes) to 26.55 (Barbosa), showing that there was no possible toxicity due to aluminum in the soil (Wright, 1989; Panda et al., 2009; Singh et al., 2017).

Quantification of limonoids by HPLC-DAD

Limonin (**1**) and nomilin (**2**) concentrations were calculated from duplicate HPLC analyses using quantification curves. Linear regression analysis showed that limonin and nomilin was linear across the range evaluated (R = 0.9997, *p*-value = 2.2e-16 and R = 0.9984, *p*-value = 2.2e-16, respectively). The limit of detection, limit of quantification, and resolution were also calculated. These values are summarized in Table S3.

Total glycosidic free limonoids content was estimated from the sum of limonin and nomilin (Tian et al., 2007; Liu et al., 2015) (Fig. 1 and Figure S2). The content in seeds was highest for the A-O sample (7497 ± 870 mg/kg), equivalent to 0.75%, and lower for A-A (1035 ± 140 mg/kg), equivalent to 0.1%. The nomilin/limonin ratio was greater than 1 in the A-L and A-O samples, indicating that they had a higher amount of nomilin than limonin, as shown in Fig. 2 and Table 1. Analysis of variance of the means of limonoids content (mg/kg seed) showed that at least one pair of means was different between the samples (value $p < 0.05$) and Tukey's HSD was used to determine differences between pairs of means (Table S4).

Identification of limonoids by HR-MS

Chromatograms of A-O extracts had peaks at the retention times of limonin and nomilin (Figure S3). The spectra obtained by ESI-HR-MS in positive mode had an ion at *m/z* 471.2012, which corresponded to the pseudomolecular ion $[M+H]^+$ for limonin $[C_{26}H_{30}O_8]^+$, which has a calculated molecular weight of 471.2013 g/mol. A peak with *m/z* 515.2273 represents the pseudomolecular ion $[M+H]^+$ of nomilin $[C_{28}H_{35}O_9]^+$, which has a calculated molecular weight of 515.2275 g/mol, as shown in Figure S4.

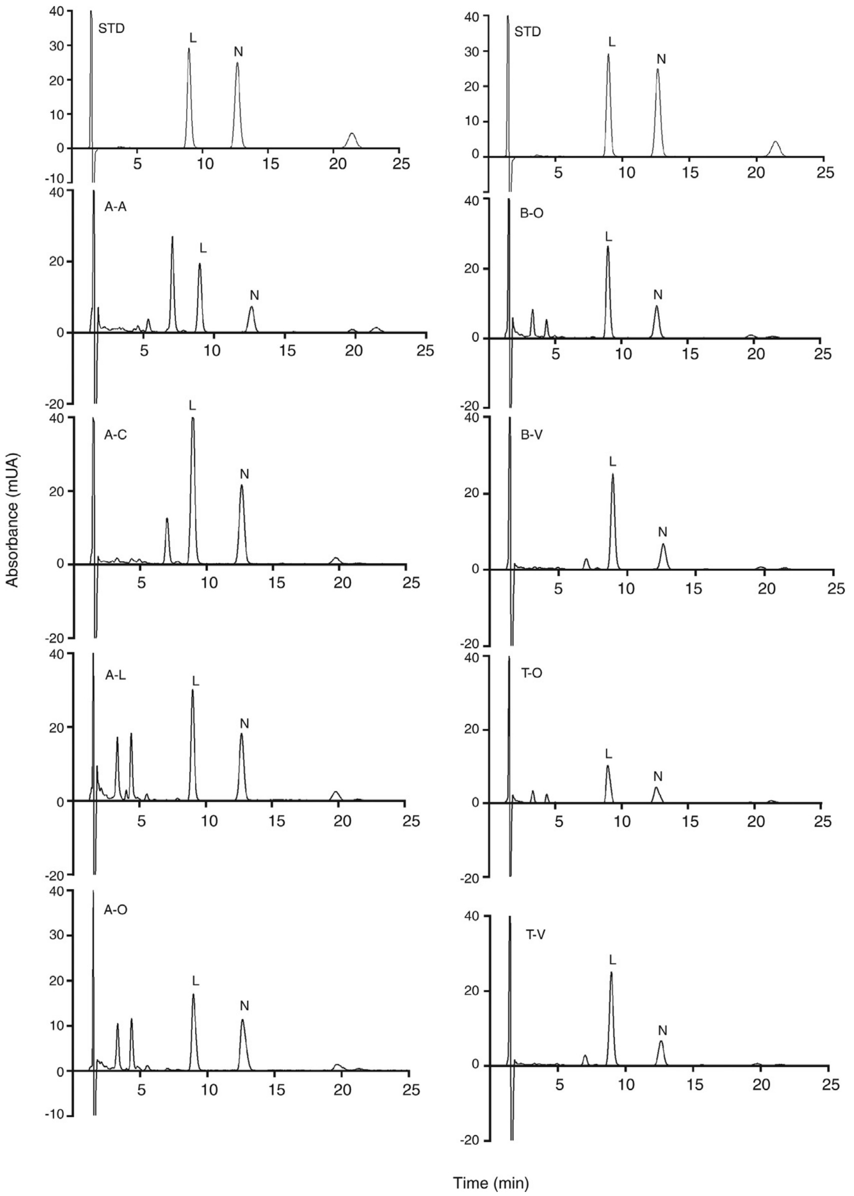

Fig. 1. HPLC-DAD profiles of seeds extracts. Extracts of all the *Citrus* samples studied were prepared from dried dichloromethane extract in the mobile phase at a concentration of 500 μg/ml and analyzed by HPLC to compare its content of limonin (**1**) and nomilin (**2**). The limonin and nomilin standards was prepared at 200 μg/ml in the mobile phase. STD: standard. A-A: Sour orange from Andes; A-C: Criolla orange from Andes; A-L: Tangerine-lemon from Andes; A-O: Oneco tangerine from Andes; B-O: Oneco tangerine from Barbosa; B-V: Valencia orange from Barbosa; T-O: Oneco tangerine from Támesis; V-T: Valencia orange from Támesis.

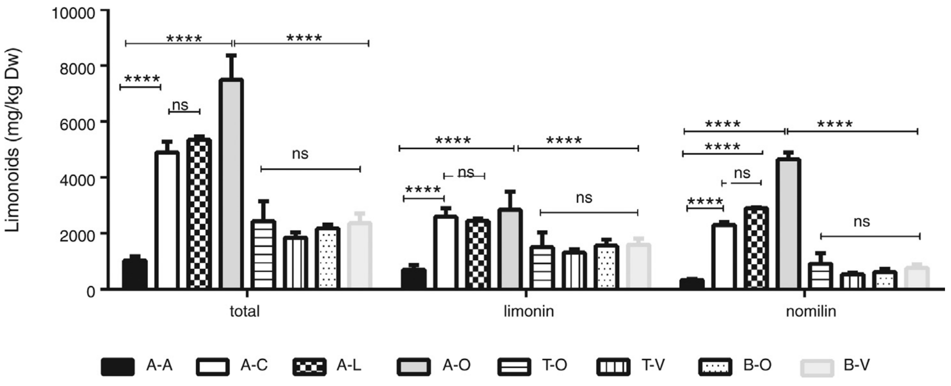

Fig. 2. Content of glycosidic free limonoids in *Citrus* seeds. The content of limonin, nomilin, and total LA expressed like mg/kg dry seed, was estimated from the dichloromethane extract in triplicated of each citric sample and its relation with the starting weight of dry seed used (1–3) g. Bars represent the mean ± SD of limonin and nomilin. $^{****}p < 0.0001$, ns (no statistical significance). A-A: Sour orange from Andes. A-C: Criolla orange from Andes. A-L: Tangerine-lemon from Andes. A-O: Oneco tangerine from Andes. B-O: Oneco tangerine from Barbosa. B-V: Valencia orange from Barbosa. T-O: Oneco tangerine from Támesis. T-V: Valencia orange from Támesis.

Table 1
Quantification of citric limonoids aglycone in the Department of Antioquia.

Municipality	Common name	Limonin	Nomilin	N/L	Total limonoids mg/kg (%)
Andes	Oneco tangerine	2846 ± 649	4651 ± 248	1.68 ± 0.32	7497 ± 870 (0.75)
	Tangerine-lemon	2447 ± 86	2902 ± 26	1.19 ± 0.03	5349 ± 112 (0.53)
	Criolla Orange	2598 ± 309	2301 ± 116	0.89 ± 0.10	4899 ± 385 (0.49)
	Sour Orange	701 ± 160	334 ± 35	0.50 ± 0.13	1035 ± 140 (0.10)
Barbosa	Valencia Orange	1600 ± 221	759 ± 135	0.47 ± 0.02	2360 ± 354 (0.24)
	Oneco tangerine	1564 ± 223	619 ± 115	0.41 ± 0.14	2183 ± 142 (0.22)
Támesis	Oneco tangerine	1510 ± 526	923 ± 369	0.64 ± 0.24	2433 ± 717 (0.24)
	Valencia Orange	1316 ± 126	541 ± 60	0.41 ± 0.01	1856 ± 184 (0.19)

Total limonoid content obtained from the average of three extractions ± SD.

Discussion

In this study, using ESI-HR-MS in positive mode, we observed an ion at *m/z* 471.2012, which corresponded to limonin, and another at *m/z* 515.2273, which corresponded to nomilin, These results were similar to those reported in a previous study (Manners and Breksa, 2004).

The HPLC-DAD method showed good selectivity and a resolution of 1.92, in accordance with AOAC (Association of Official Agricultural Chemists) specifications. The seeds A-O had the highest quantity of limonoids (7494 ± 870 mg/kg seed), followed by A-L (5349 ± 112 mg/kg seed), which represents 0.75% and 0.53% (w/w), respectively. In B-O and T-O, the LA content was between 0.22 % and 0.24 % (w/w), which was lower than the content found in the A-O sample. In 3 of the 4 farms sampled, tangerines presented a higher content of total LA compared to oranges, as previously reported by Rouseff and Nagy (1982), tangerines are distinguished from other species by virtue of having the highest concentration of limonin, nomilin, and total limonoids.

The content of limonoids in seeds of T-V and B-V collected in November 2017 in the municipalities of Támesis and Barbosa was between 1856 ± 184 mg/kg (0.19%) and 2433 ± 717 mg/kg (0.24%). Rouseff and Nagy (1982) reported a concentration of 2136 mg/kg (0.21%), which was the sum of limonin, nomilin, deacetyl nomilin, and obacunone. Gerolino et al. (2015) used a colorimetric method to determine total LA in *C. sinensis* (Pera-Rio), which resulted in a reported value of 4000 mg/kg, which was higher than the value found in our study.

The A-A sample had the lowest amount of LA among all the extracts studied (1035 ± 140 mg/kg (0.10%). This value was similar to that reported by Rouseff and Nagy (1982) for *C. aurantium.* They reported a value of 2605 mg/kg, of which 42.5% was deacetyl nomilin and ubacunone, and 57.5% was limonin and nomilin, resulting in 1261 mg/kg limonin and nomilin. This extract also contained another peak that eluted after limonin with a retention time of approximately 7 min with a greater area count than that for both limonin and nomilin (Fig. 1).

The chromatograms of Sour, criolla, and Valencia orange showed similar patterns. These patterns differed from the chromatographic patterns of tangerine-lemon and Oneco tangerine, which showed minor peaks that eluted after limonin (Rouseff and Nagy, 1982). Limonoid content was not significantly different between the *Citrus* fruits harvested in the municipalities of Barbosa and Támesis for Valencia orange and Oneco tangerine. Taking into account that the *Citrus* fruits of the Támesis municipality are from plantation, while those of Barbosa they are wild trees, the type of crop has no effect on the concentration of limonoids in seeds as reported Rouseff and Nagy (1982).

By normalizing the content of nomilin to that of limonin (**2**/**1**), 25% of the samples (A-O and A-L) had higher levels of nomilin than limonin (Table 1). Nomilin is important because it is a triterpenoid precursor that is converted to other limonoids through oxidation, isomerization, methylation, acetylation and hydrolyzation (Hasegawa et al., 1980; Ou et al., 1988; Wang et al., 2017). In this study, all the extracts of the evaluated *Citrus* samples contained both limonin and nomilin, suggesting that the seed has characteristics suitable for use as an elite material for evaluation of limonoid production by biotechnological processes, since it is an excellent source of LA (Wang et al., 2016).

The methodology used in this investigation allowed for extraction, quantification by HPLC-DAD, and identification by ESI-HRMS of limonin and nomilin in extracts of *Citrus* seeds belonging to the Rutaceae family, a group of plants with promising antiparasitary activities. The A-O seeds, an important source LA, can be used as an elite material for tissue culture and to evaluate limonoid production by biotechnological processes. Increased production of limonoids will allow for these compounds to be available for all phases of research, innovation, and drug development.

Authorship

CM: MSc student, laboratory work, extraction and quantification of limonoids by HLPLC-DAD, data analysis and draft of the article. LG: Identification of glycosidic free limonoids by HR-MS, critical reading of the article. SR: Advisor of MSc student, study design and critical reading of the article. LA and SP: Study design and critical reading of the article.

Conflict of interest

The authors declare no conflicts of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.07.006.

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