



Limonoid detection and profile in callus culture of sweet orange

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ABSTRACT. Plant tissue culture has emerged as an important tool to produce bioactive compounds from various plant species, including the sustainable production of limonoids that are receiving considerable attention due to the benefits associated with human health such as anticancer activities. The purpose of the present study was to evaluate the capacity of limonoids aglycone production from callus culture from sweet orange cv. Pera (*Citrus sinensis*) seeds

and identify the compounds produced in this cell line. Callus induction occurred in Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic (2,4-D), malt extract, agar and coconut water. For the analysis and identification of the limonoids, CG-MS-EI ion-positive mode and UPLC-QTOF-ESI were used operating in positive and negative mode. An intense peak corresponding to limonin appeared in the callus extracts at a retention time of 58.1 min. in CG-MS-EI and four major limonoids aglycone by positive ion mode UPLC-QTOF-ESI: limonin, nomilin, deacetylnomilin, and nomilinic acid. The culture medium was efficient at the bioproduction of limonoids aglycone in callus cultures of *C. sinensis* seeds. Therefore, data obtained from UPLC-QTOF-ESI proved its importance at identifying new compounds that benefit human health, and may assist future work in the identification of known or new limonoids in *Citrus* species and related genera.

Keywords: seeds; MS medium; limonoids; CG-MS-EI; UPLC-QTOF-ESI.

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Introduction

The production of original and/or modified bioactive compounds through living organisms is a major goal of biotechnology. In plants, these compounds are present in small quantities and exert important biological activities for human health, being fundamental in the process of discovery and development of new drugs (Velu, Palanichamy, & Rajan, 2018). It is estimated that 35% of therapeutic medicines are of natural origin (Calixto, 2019), therefore, biotechnological techniques, such as *in vitro* plant cultivation, aim to increase the bioprospection of these compounds, by preventing the loss of biodiversity through the use of few natural sources, decreasing unsustainable extractivism and contributing to species conservation (Souza, Rescarolli, & Nunez, 2018).

Callus production consists of a plant tissue culture technique aimed at genetic enhancement and the production of bioactive compounds from many woody plant species, including the genus *Citrus* (Fallico, Ballistreri, Arena, Brighina, & Rapisarda, 2017). This technique offers a defined production system and is not subject to climatic and soil influences for being conducted in a controlled environment, which allows it to be applied in biomass production and development of somaclonal variations and exploited through biotechnological production means for new cultivars (Gonçalves & Romano, 2013).

Among the commercial species of *Citrus*, we highlight sweet orange, used in large quantities to produce juices, generating numerous residues that can be harmful to the environment (Ho & Lin, 2008). Among these residues, peels and seeds can be used as alternative sources of bioactive compounds such as flavonoids and limonoids (Avula et al., 2016; Zou, Xi, Hu, Nie, & Zhou, 2016; Celano et al., 2018; Russo et al., 2019).

Limonoids are highly oxygenated triterpenes with a bitter taste and low water solubility and can be obtained from citrus seeds in significant amounts in their natural or glycosylated form (Figure 1) (Manners &

Brekša, 2004; Zukas, Brekša, & Manners, 2004; Liao, Xu, Liu, & Wang, 2012). Studies have demonstrated the medicinal activities of limonoids, for example, inhibiting carcinogenesis (Langeswaran, Jagadeesan, Revathy, & Balasubramanian, 2012; Liao et al., 2012), HIV-1 (Battinelli et al., 2003), as antioxidants (Hamdan et al. 2011; Zou et al., 2016), in addition to antimalarial (Bray, Warhurst, Connolly, O'Neill, & Phillipson, 1990), anti-inflammatory (Mahmoud, Hamdan, Wink, & El-Shazly, 2013; Yang et al., 2014), and antibacterial (Ribeiro et al., 2008; Vikram, Jayaprakasha, Jesudhasan, & Pillai, 2012) activities.

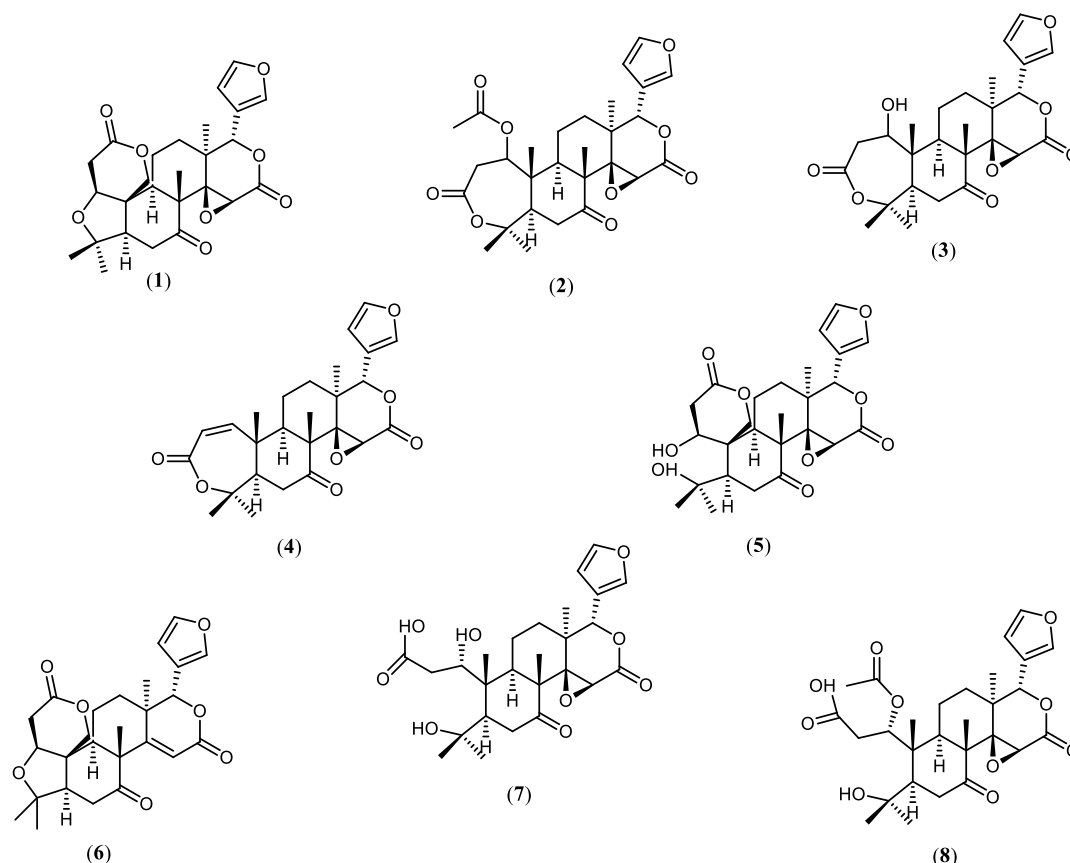


Figure 1. Main limonoids aglycone isolated in *Citrus* species (Manners, 2007). Limonin (1), Nomilin (2), Deacetylnomilin (3), Obacunone (4), Ichangin (5), Deoxylimonin (6), Deacetylnomilinic Acid (7), Nomilinic Acid (8).

Because of their benefits to human health, several studies have sought to identify limonoids in citrus plants through different analytical techniques (Manners, Hasegawa, Bennett, & Wong, 2000; Tian, Li, Barbacci, Schwartz, & Patil 2003; Gerolino et al., 2015; Avula et al., 2016). However, few studies report the behavior of callus culture in *C. sinensis*, as well as its capacity to produce limonoids (Gerolino et al, 2015).

Therefore, the objectives of this study were to evaluate the ability of limonoid aglycone bioproduction from calluses from *C. sinensis* seeds and identify the compounds present in this cell line through Gas Chromatography coupled to Mass Spectrometry by electronic impact ionization (CG-MS-EI) and Ultra-Efficient Liquid Chromatography coupled to high-resolution quadrupolar-flight time analyzer (UPLC-QTOF-ESI) mass spectrometry.

Material and methods

Plant material

Ripe seeds of sweet orange cv. Pera were purchased from a supplier in the municipality of Paranavaí, located in the northwest of Paraná state, Brazil. For *in vitro* cultivation, the seeds were subjected to a disinfection process before using them in the callus induction. The seeds were briefly peeled and sterilized for 15 min. in 0.5% (w v⁻¹) sodium hypochlorite, after with 0.1% (w v⁻¹) Folicur® antifungal solution, and washed in sterile distilled water before being inoculated into the culture medium. The culture medium used was MS (Murashige & Skoog, 1962) supplemented with 30 g L⁻¹ sucrose (w v⁻¹), 2.0 mg L⁻¹ 2,4-

dichlorophenoxyacetic acid (2,4-D), 500 mg L⁻¹ malt, agar (0.8%) (w v⁻¹), and 200 ml l⁻¹ commercial coconut water (*Sococo*[®]). After preparation, it was transferred to the culture medium to glass flasks and autoclaved for 20 min. at 121°C. Subsequently, sterile orange seeds were introduced into the glass flasks and incubated at 28°C in darkness for induction and callus formation.

After the fourth subculture generation (120 days), *C. sinensis* callus were lyophilized and subjected to the following extraction process: 1 g of the lyophilized callus sample were subjected to hexane extraction in the Soxhlet apparatus to remove the fixed oils, then with acetone to obtain the limonoids aglycone. After extraction, the acetone extract was taken to the rotary evaporator to obtain dry extracts and subsequently stored at -20°C up to GC-MS-EI and UPLC-QTOF-ESI analysis.

Gas-impact chromatographic analysis coupled to electron impact mass spectrometry (GC-MS-EI)

The acetonetic callus extract was subjected to gas chromatography-mass spectrometry (GC-MS) analysis at the *Universidade Unicesumar* (Maringá, Paraná state, Brazil) laboratory using the Agilent[®]-7890B gas chromatograph equipped with a phase capillary column. Stationary: Agilent HP-5MS-IU (30 m x 0.250 mm x 0.25 µm) and Agilent[®]-5977-A mass spectrometer. Samples were injected into a GC/MS equipped with a phenyl-methyl-silicon column DB5 (30 m x 0.25 mm, 0.25 µm film thickness) interfaced with a mass spectrometer. The oven temperature was set at an initial temperature of 200°C and increased to 310 at 2°C min.⁻¹. Injector temperature and transfer line temperature were 400°C and ions source temperature 250°C. The carrier gas was helium, with linear flow set to 1 mL min.⁻¹; split rate, 1:30; ionization energy 70 eV and in the full scan mode.

UPLC analysis coupled to high resolution electrospray ionization mass spectrometry (MS-ESI)

It was employed liquid chromatography system Acquity UPLC, in which the separation was performed on a CSH C18 Waters column (2.1 x 100 mm, with 1.7 µm particles and 135 Å pore). The mobile phase consisted of H₂O with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), which were used as the following elution gradient 5% B of 0-1 min., 50% B 5-10 min., 70% B 10-12 min., 98% B 12-14 min, maintained at 98% B 14-18 min., then 5% B at 18-20 min., which was maintained at 20-15 minute. The sample was solubilized in 1000 µl of an acetonitrile: H₂O mixture (1:1, v v⁻¹). The column temperature was 40°C at a flow rate of 0.25 mL min.⁻¹.

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a Quadrupole-Flight Time Geometry Mass Spectrometer (Impact II-Bruker) equipped with an electrospray ionization source (ESI). The mass spectrometer was operated in positive mode and negative ionization mode. The experimental conditions were as follows for the positive mode; capillary voltage 4500 V, capillary temperature 180°C, scan range 50-1200 m z⁻¹. For the negative mode were capillary voltage 4500 V, capillary temperature 180°C, scan range 50-1300 m z⁻¹. Limonoids were identified by comparison with literature data and by their exact mass determination, with a mass error calculation below 10 ppm (parts per million).

Results and discussion

Callus culture

MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), malt extract, and coconut water showed good callus biomass development during a 120-day cultivation, to achieve genetic stability, and may have the ability to biosynthesize the limonoids. For this reason, a culture medium similar to Gerolino et al., (2015) was used, with minor modifications: malt extract addition, coconut water, and different 2,4-D concentration.

GC-MS-EI limonoids analysis

After reaching stability, we proceeded with *C. sinensis* callus extraction with acetone and analysis through GC-MS-EI, which almost only showed the presence of the limonin (1), the major limonoid aglycone found in the genus *Citrus* (Figure 2), which can be identified by comparison of its mass spectrum with NIST Library spectra and limonin fragmentation mode (Ren et al., 2014). Figure 2A shows the GC-MS chromatogram of callus acetone extract showing the intense peak corresponding to limonin (1) at a retention time of 58.1 min. The smaller peaks near limonin (1) may be associated to other limonoids with similar structures that could not be identified by this methodology

Tian et al. (2003) provided the literature with good descriptions of electron impact mass spectrometry (MS-EI) data from the four most common citrus limonoids aglycone obtained from citrus fruit seeds, limonin (1), nomilin (2), deacetylnomillin (3), and obacunone (4). As this spectrum was obtained with an ion source at EI of 70 eV, limonin (1) (Figure 2) did not show a molecular ion, but exhibited at m/z 95, the fragment-ion characteristic of *Citrus* limonoids, that is associated with the presence of the furan ring. Other important fragment ion is at m/z 347, reported as the base peak in the limonin spectrum (1), it was possibly produced by the elimination of the furan ring and further elimination of one molecule of carbon monoxide, $[470-95-CO]^+$, after that, the ion at m/z 329 produced from the loss of a water molecule $[347-H_2O]$ and ion at m/z 455 formed from the loss of a methyl radical (Figure 2B) (Tian et al., 2003).

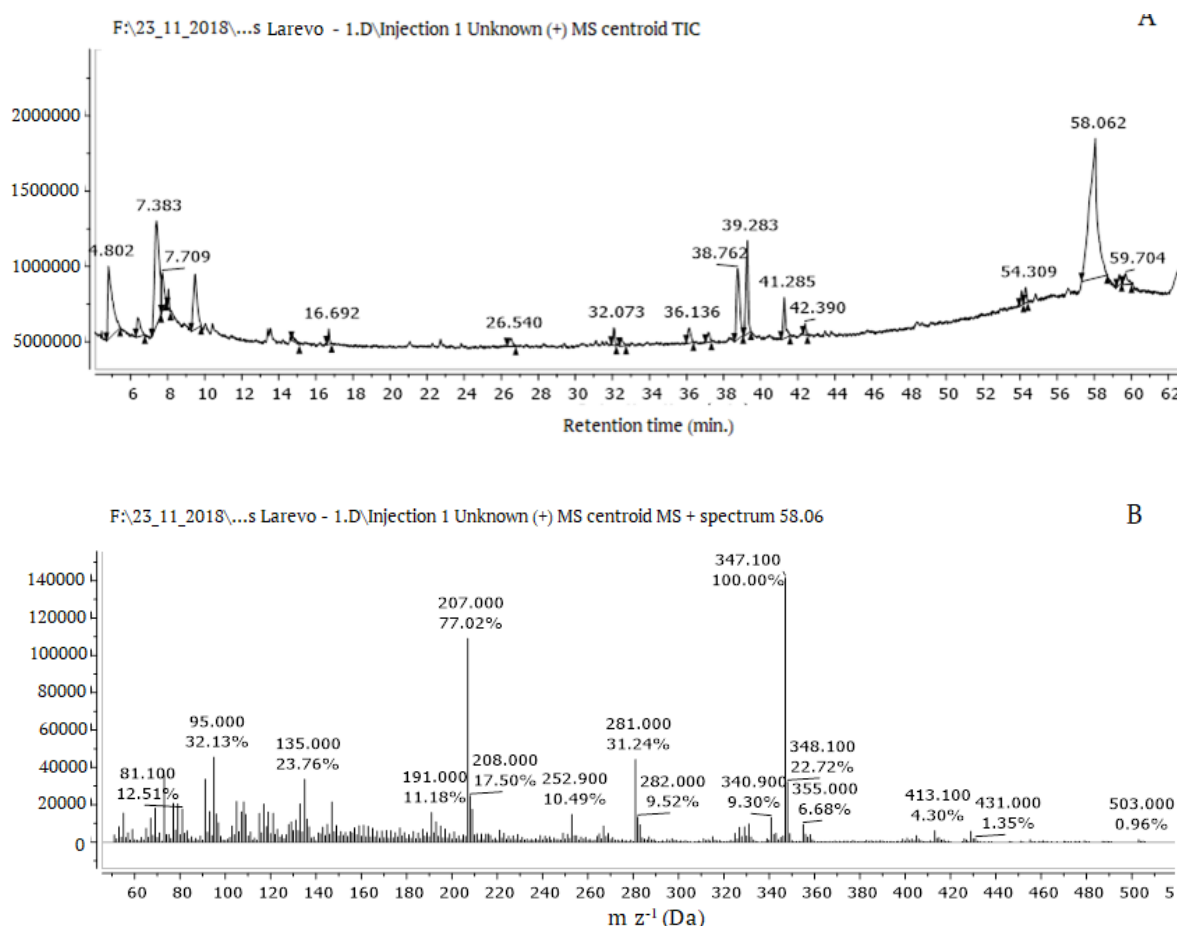


Figure 2. Total Ion Chromatogram of *Citrus sinensis* callus acetone extract obtained by GC-MS (A); limonin mass spectrum (1) (B).

UPLC analysis coupled to high resolution electrospray ionization mass spectrometry (MS-ESI)

The identification of minor limonoids aglycone in the acetonic extract was not possible using GC-MS-EI, but it could be done when acetonic callus extract was analyzed by UPLC-QTOF-ESI. The identification of each compound detected was based on its exact mass and by comparison with mass spectra of known limonoids aglycone present in *Citrus* species (Manners et al., 2000). Table 1 summarizes the results.

The limonoids: limonin (1), nomilin (2), deacetylnomillin (3) and nomilinic acid (8) were detected between 13 and 16 min. (Figure 3). These compounds exhibited characteristic limonoids ions: $[M+H]^+$ m/z 471.2007 ($C_{26}H_{30}O_8$), 515.2272 ($C_{28}H_{34}O_9$), 473.2169 ($C_{26}H_{32}O_8$), and 533.2361 ($C_{28}H_{36}O_{10}$), respectively (Figure 4).

The MS/MS spectra of limonin (1) precursor ion $[M+H]^+$ m/z 471.2007 ($C_{26}H_{30}O_8$, error -1.2733 ppm) produced $[M+H]^+$ m/z 453.1903 ions ($C_{26}H_{29}O_7$), 425.1954 ($C_{25}H_{29}O_6$), 409.2003 ($C_{25}H_{29}O_5$), and 367.1898 ($C_{23}H_{27}O_4$), attributed as losses of H_2O , CH_2O_2 , CH_2O_3 , $C_3H_4O_4$, respectively. Figure 5A shows the fragmentation pathway of limonin (1). Similarly to deacetylnomillin (3) $[M+H]^+$ ion 473.2169 ($C_{26}H_{32}O_8$, error -1.9018 ppm) produced the $[M+H]^+$ ions of m/z 455.2065 ($C_{26}H_{31}O_7$), 427.2104 ($C_{25}H_{31}O_6$), 411.2166 ($C_{25}H_{31}O_5$), and 369.2059 ($C_{24}H_{33}O_3$) with losses of H_2O , CH_2O_2 , CH_2O_3 , $C_3H_4O_4$, respectively. Neutral losses

of H₂O, CO and CO₂ were the main fragmentation patterns for limonin (1) and deacetylnomillin (3) (Figure 5B). In addition, the characteristic ion for *Citrus* m/z⁻¹ 161.0597 (C₁₀H₉O₂) limonoids was found in its MS/MS spectrum, which was attributed to the furan ring bound to a lactonic ring residue (Ren et al., 2014).

Table 1. Mass data of limonoids aglycone detected in acetone callus extract of *Citrus sinensis* using UPLC-QTOF-ESI-MS.

RT (min.)	Substance	Molecular Formula	Exact Mass	Monoisotopic Mass	Base Ion [H] ⁺ _{exp.}	Error (ppm)
14.55	Limonin (1)	C ₂₆ H ₅₀ O ₈	470.1940	471.2013	471.2007	-1.2733
15.10	Nomilin (2)	C ₂₆ H ₅₄ O ₉	514.2203	515.2275	515.2272	-0.5822
13.80	Desacetylnomilin (3)	C ₂₆ H ₅₂ O ₈	472.2097	473.2169	473.2160	-1.9018
14.26	Nomilinic Acid (8)	C ₂₈ H ₅₆ O ₁₀	532.2308	533.2381	533.2361	-3.7506

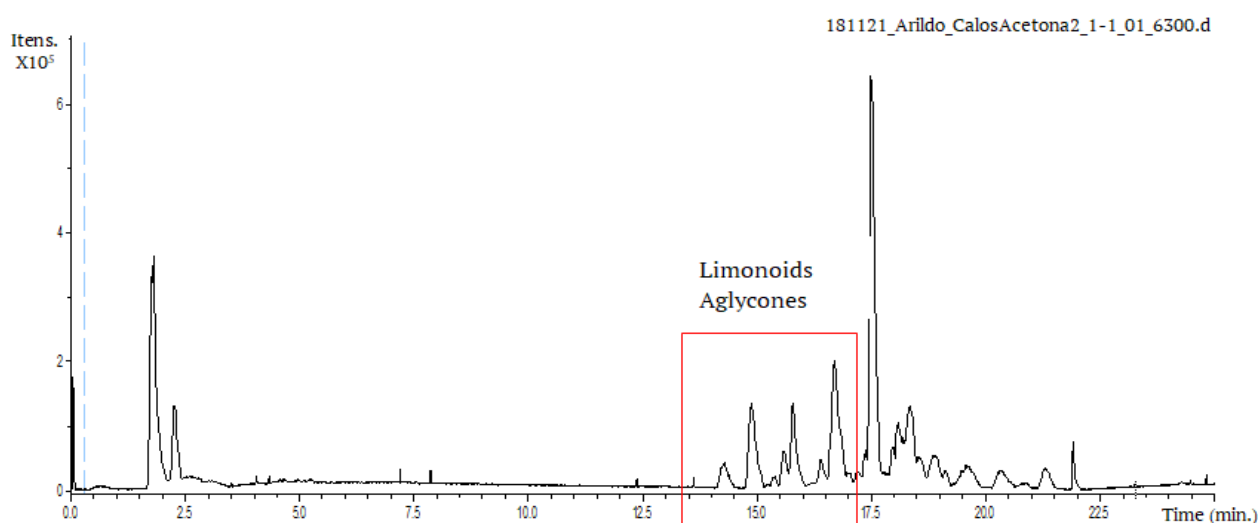


Figure 3. UHPLC-QTOF-MS chromatogram of acetone callus extract of *Citrus sinensis*, acquired in positive ionization mode.

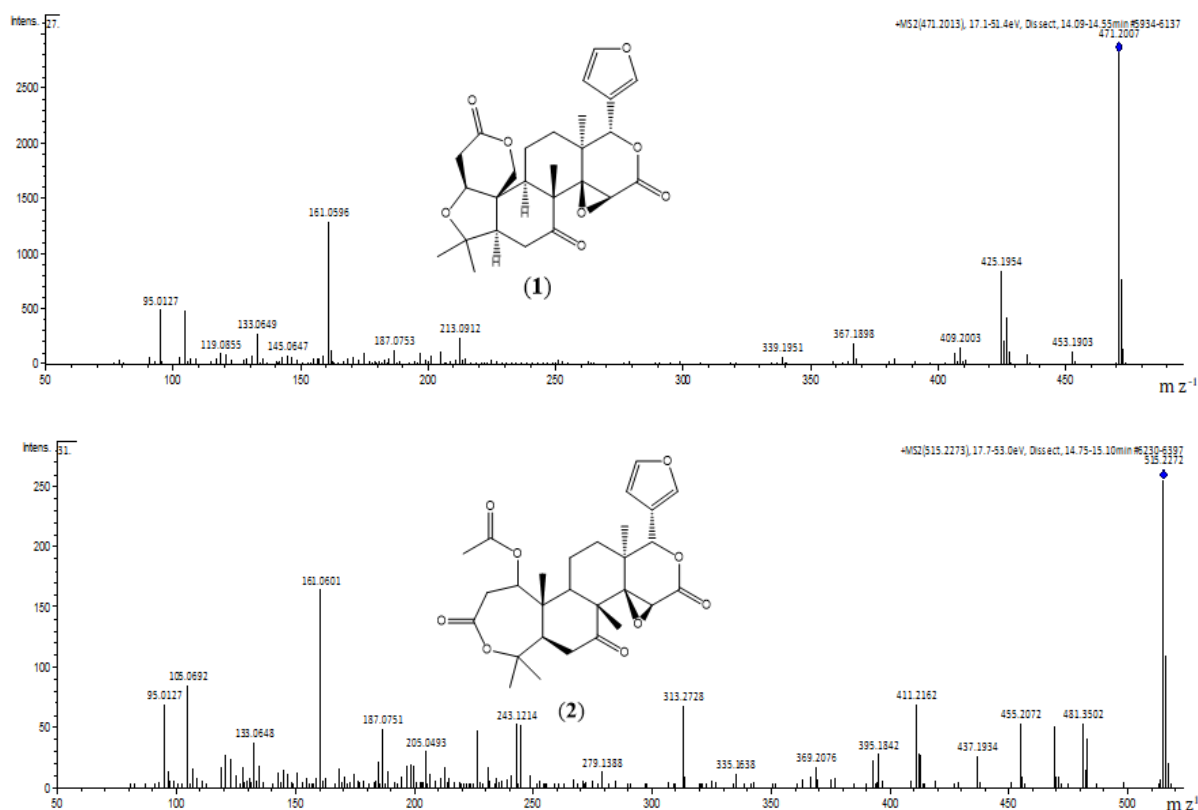


Figure 4A. Positive mode electrospray ionization mass spectra of limonin (1) and nomilin (2).

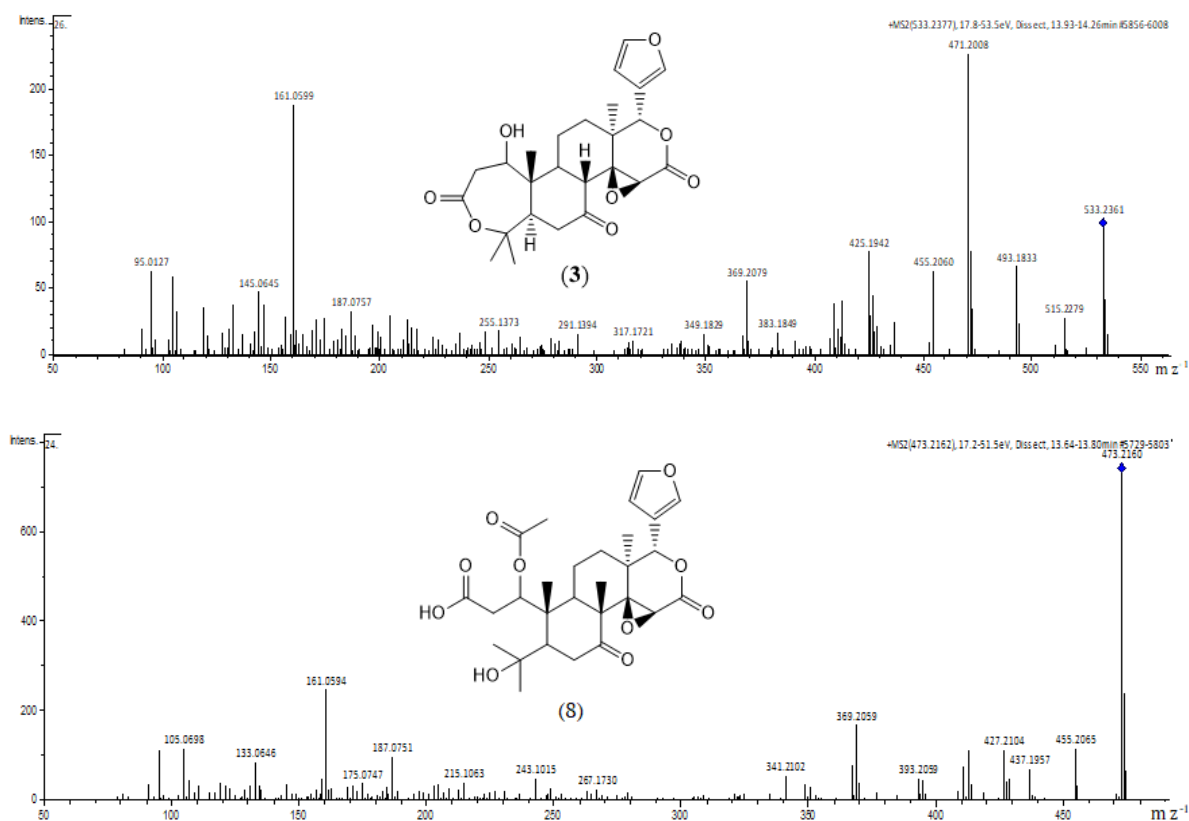


Figure 4B. Positive mode electrospray ionization mass spectra of deacetylnomillin (3) and nomilinic acid (8).

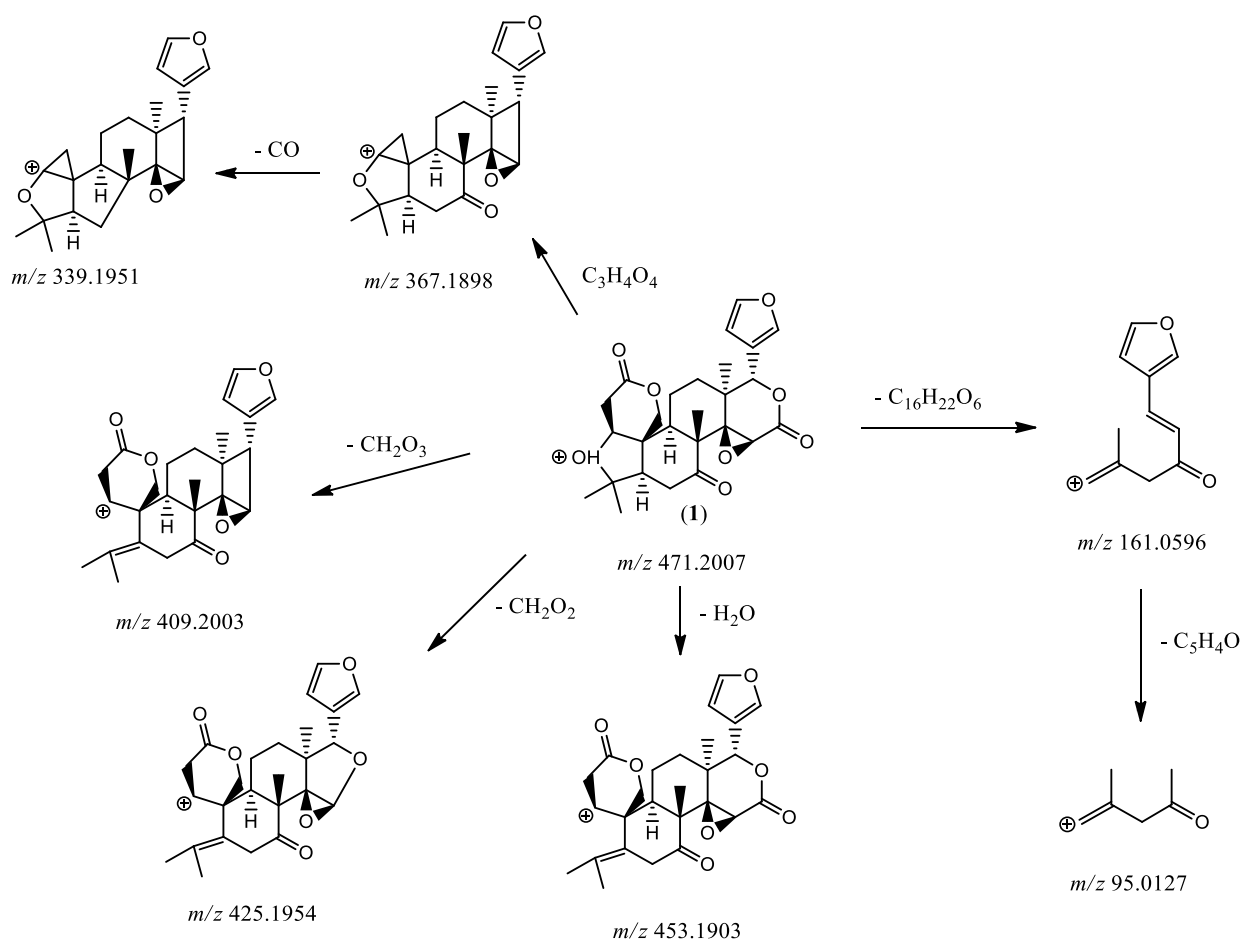


Figure 5A. Fragmentation pathway of limonin (1) proposed by Ren et al. (2014).

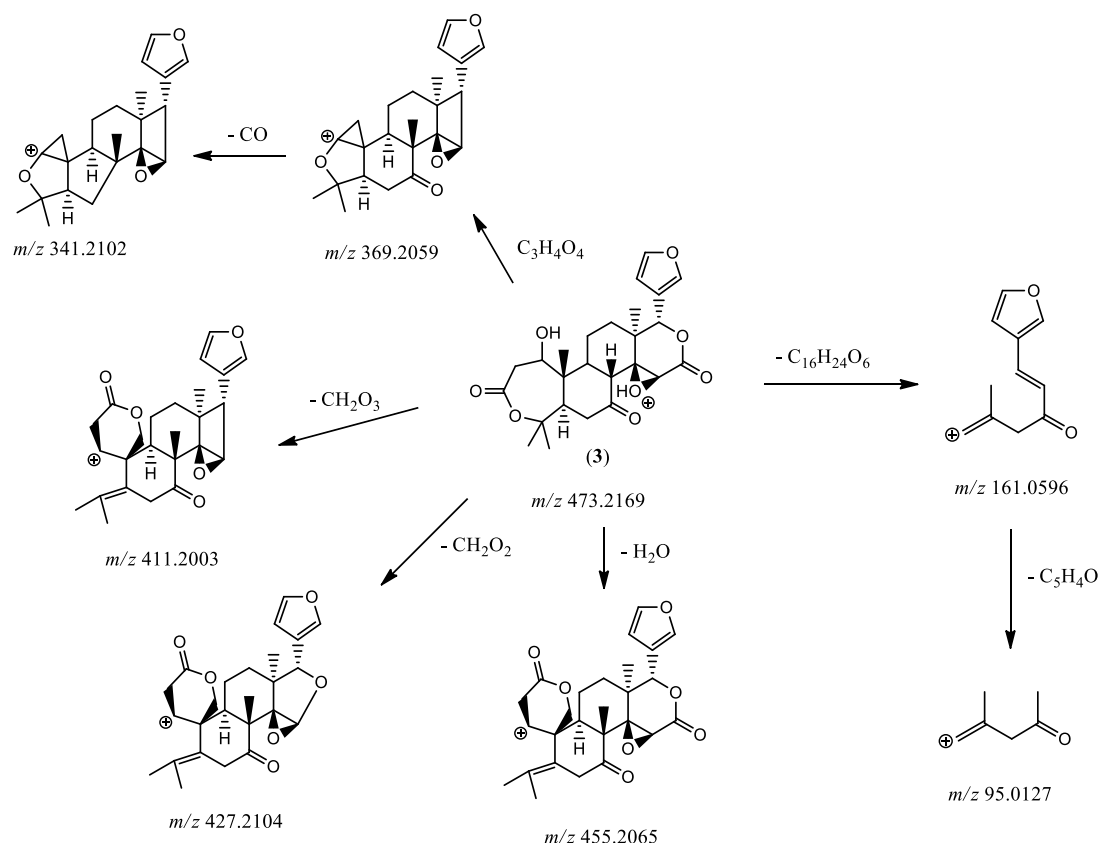


Figure 5B. Fragmentation pathway of the deacetylnomillin (3) proposed by Ren et al. (2014).

Conclusion

The culture medium used was adequate at the callus development, having maintained its biosynthetic capacity in the bioproduction of limonoids, also shown as a valuable tool to study *Citrus* limonoids biosynthesis, UPLC-QTOF-ESI was able to detect limonoids aglycone in acetone callus extract of *C. sinensis* and furthermore, the use of this methodology to identify known or new bioactive limonoids may be important to discover new compounds beneficial to human health and assess their biological activities.

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