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Synthesis and characterization of phloroglucinol derivatives

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Trabalho de Conclusão de Curso apresentado à Universidade Federal do Rio Grande do Sul (UFRGS) como parte das exigências para a obtenção de Título de Farmacêutico.

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1 Abstract

Phloroglucinol derivatives have been proved to have great pharmacological activity. Two acylated phloroglucinol were synthesized by Friedel-Crafts reaction and characterized by High Performance Liquid Chromatography (HPLC), Differential Scanning Calorimetry (DSC) , mass spectroscopy and Nuclear Magnetic Resonance (NMR) . The one-step synthesis was considered simple, giving a yield for 68% of monoacylphloroglucinol (MAPG) and 32% for diacylphloroglucinol (DAPG). Purification by crystallization of DAPG was more efficient than column chromatography of MAPG, showing great results after analysis. The analytical methods used in this work have proven to be simple and efficient to characterize both compounds, resulting in important information to proceed in the search of novel therapies.

Keywords: *monoacylphlorogluciol, diacylphloroglucinol, synthesis, NMR, HPLC, DSC.*

2 Introduction

For many years, the search for new bioactive compounds is a constant challenge in the pharmaceutical field, and plants are its main source. The drugs synthesized nowadays were at some point inspired by natural substances (eg. Paclitaxel from *Taxus bacatta*, salicylic acid from *Salix alba*, Artemisin derivatives from A*rtemisia annua* etc.) (Veeresham et al. 2012). Phloroglucinol derivatives are secondary metabolites found naturally in certain plant species from the genus Hypericum (Ccana-Ccapatinta et al.2015; Dall'Agnol 2005; Franca 2013). Many studies have proven that some phloroglucinol derivatives present great pharmacological effect, such as antidepressant (Ccana-Ccapatinta et al.2015), antitumoral (Huang 2011), antimicrobial (Dall'Agnol 2005) and antinflamatory (Ishii 2002) activities. These finds show good potential for this class of polyphenols and drive to a better understanding of novel therapies.

Extraction of these compounds is time and effort-demanding and most of the times gives a poor yield (Nunez, P.N; et al 2015; Abe, S.; Tanaka, N; and Kobayashi, J. 2012). Synthesizing them drives to a better effective way to obtain great amounts of phloroglucinol derivatives. Based in reported activities of plant-derived phloroglucinols (Ishii 2002; Dall'Agnol 2005; Magalhães 2010; Huang 2011; Veeresham et al. 2012; Franca 2013; Ccana-Ccapatinta et al.2015), two acylated phloroglucinols were synthesized by Friedel-Crafts reaction and subsequently characterized by HPLC-DAD, DSC, LC-MS and NMR.

3 Experimental

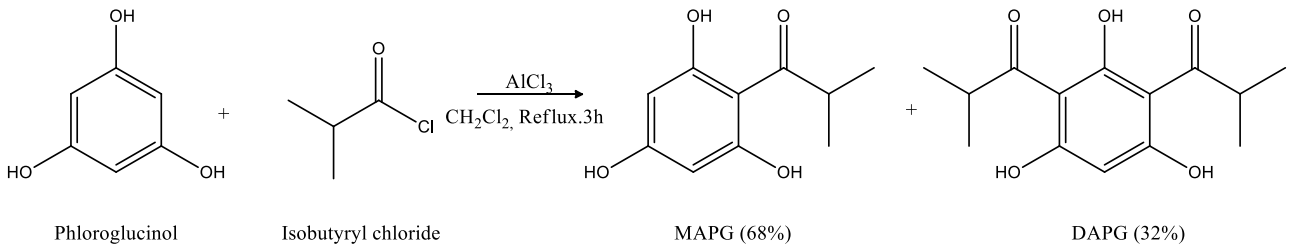
3.1 Synthesis Section

Materials

All chemicals were purchased from Sigma-Aldrich, Synth and Nuclear. Analytical grade dichloromethane was used for synthesis. Cyclohexane and Ethyl acetate were used for column chromatography. Analytical grade acetonitrile was used for HPLC.

Synthesis of diacylated phloroglucinol with Isobutyryl chloride

To a round-bottom flask were added respectively: 100 mg of phloroglucinol (0.79 mmol), 265 mg of aluminium chloride (2 mmol; 2.5 eq) and dry dichloromethane until total solubilization. The reaction was stirred at room temperature, under nitrogen atmosphere (Scheme 1). After 15 minutes, 84,4 µL of Isobutiryl chloride (0.86 mmol; 1.1 eq) was added into the reaction flask, in cold bath. After the addition of the last reactant, the temperature was risen and the reaction was kept under reflux for 3 h. Gradually, the reaction, which starts with a yellow coloration, became brownish. The reaction was monitored by Thin Layer Chromatography (TLC). The reaction was stopped by addition of HCl 4 M and a precipitate was formed. The product was pounder into a flask with an ice cube and the precipitate (DAPG) was washed with cold water until obtaining a white crystalline powder (20% yield). The filtered was extracted three times with ethyl acetate, and dried with anhydrous sodium sulfate. The resulting organic phase was evaporated under vacuum and the orange solid was further purified by silica gel 60 (63-200 µm) column and cyclohexane:ethyl acetate (99:1, *v/v*), to give the monoacylated phloroglucinol (MAPG) as a majority product (68% yield) and diacylated phloroglucinol (DAPG) as a minority (12% yield).



Scheme1.Phloroglucinol acylation using Friedel-Crafts.

Purification of Monoacylated phloroglucinol

Monoacyl phloroglucinol was purified using column chromatography. The chromatography was carried out over silica gel 60 (63-200 μm) using a mixture of cyclohexane:ethyl acetate (99:1, *v/v*) as mobile phase. The samples were monitored by TLC and the polarity of the mobile phase was gradually increased, reaching a final ratio of cyclohexane: ethyl acetate (90:20, *v/v*).

3.2 Characterization Section

Samples preparation

Suitable amount of DAPG and MAPG were weighed and dissolved in 20 mL of acetonitrile/ water (1:1, *v/v*) to obtain a sample with final concentration of 100 μg/mL.

HPLC Characterization

The HPLC analysis were performed in a Shimadzu (Kyoto, Japan) system consisting of a pump (LC-20AT), a diode array detector (SPD-M10A), a system controller (CBM-20A), an autoinjector (SIL-20A). Class-VP 6.14 SP2 software controlled the HPLC system, data acquisition and processing. A Phenomenex® (Torrance, CA, USA) Kinetex C18 column (100x4.6 mm, i.d. 2.6 μm, 100 A°) kept at room temperature was used for HPLC analysis of DAPG and MAPG. The flow rate was set at 0.6 mL/min; injection volume of 10 μL, mobile phase composed of A (aqueous phosphoric acid 0.07%, v/v) and B (acetonitrile). The gradient elution with running time are described in Table 1. For peak purity analysis, spectra in the range of 210–370 nm were recorded at a frequency of 0.64 Hz and noise spectrum and background compensation subtracted from the peak spectra. The slope sensitivity for peak integration adjusted to 10,000 UV/min.

Table 1. Gradient Conditions and summary of chromatographic results.

Compound	Mobile Phase	Time run (min)
DAPG	0-10 min 57% B, 10-47 min 95% B	50 min
MAPG	0-10 min 32% B, 10-47 min 95% B	

NMR Characterization (H1 NMR and C13 NMR)

NMR spectra of both compounds, DAPG and MAPG, were obtained using Bruker Ascend^{TM} 400 MHz spectrometer. To perform the analysis, DAPG and MAPG were dissolved in deuterated Chloroform with TMS (internal Standard) or Acetone with TMS. Data analysis was processed with NMR softwares Bruker Top Spin 3.2 and Mestre Nova.

Differential Scanning Calorimetry (DSC)

Thermal analysis of the compounds was carried out under the flow of nitrogen using DSC-60 Shimadzu coupled with a Flow controller (FC-60A) and a Thermal Analysis Workstation (TA-60WS). Parameters were settle with flow rate of 5 $^{o}C.min^{-1}$ and maximum temperature of 300 ^{o}C. Data analysis was processed using software TA Acquisition 2.20 from Shimadzu Corporation.

Mass Spectroscopy

Electrospray Ionization Mass Spectrometry (ESI-MS) was carried out with LC-MS system (Infinity 1260 modules and a 6120 quadropole detector) Agilent Technologies (Santa Clara, CA, US). Each sample (50 $\mu g.mL^{-1}$ of DAPG or MAPG) was injected (20 µL) in Flow Injection Analysis (FIA) mode at a flow rate of 0.1 $mL.min^{-1}$, mobile phase consisted of acetonitrile: water (7:3, *v/v*).The mass detector was operate in negative mode (capillary voltage of – 3 kV), drying gas flow of 10 $L.min^{-1}$, nebulizer pressure of 45 psig, drying gas temperature of 350 °C, mass range of 100 – 300 m/z, fragmentor at 50 V.

4 Results and Discussion

4.1 H^1 NMR spectral features

The H^1 NMR spectra of DAPG and MAPG are shown in Figure 1. The molecule structure with respective hydrogen assignments was disposed to give a better understanding of the analysis. Analyzing both spectra, the protons from the equivalent methyl groups are centered around 1.15 ppm, with DAPG integrating twelve protons and MAPG integrating only six hydrogens, due to the number of methyl groups. The next characteristic signal is centered around 3.95 ppm, a septet of the equivalent carbons 9 and 10 for DAPG, and 8 for MAPG. The signal around 5.9 ppm, consisted in a singlet which corresponds to the proton from the aromatic ring. The signal centered in 7.25 ppm from DAPG NMR corresponds to deuterated chloroform while the signal around 2 ppm from MAPG NMR corresponds to deuterated acetone.

The resulting diacylphloroglucinol (DAPG) was also synthesized by Qian Yu and colleagues (Yu et al. 2016) to test its antifungal activity, and the ^{1}H NMR data obtained was similar to our

product. The monoacylated phloroglucinol had been isolated initially fom *Humulus lupulus*, showing an interesting anti-inflamatory activity (Bohr et al. 2005). The H^1 NMR data (Bohr et al. 2005), fit very well with ours. The remaining signals from MAPG are considered small impurities such water which did not interfere with the product characterization.

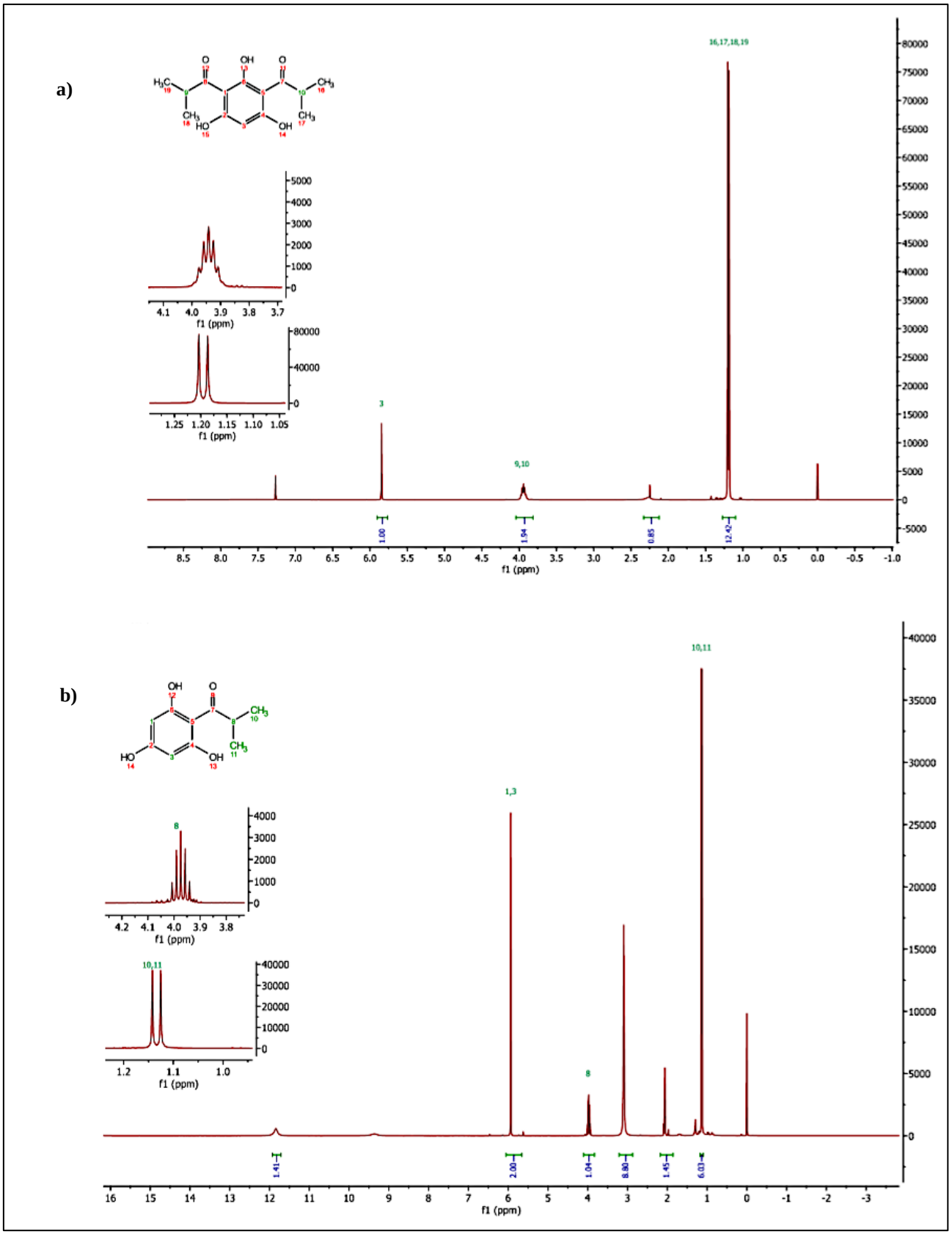


Figure 1.a) H^1 NMR spectrum of diaacylphloroglucinol (DAPG) in $CDCl_3$;b) H^1 NMR spectrum of monoacylphloroglucinol (MAPG) in Acetone-d_6

4.2 ^{13}C NMR spectral features

The ^{13}C NMR spectra of DAPG and MAPG are shown in Figure 2. In the APT mode it's possible to assign each carbon according its own proton attachment. The APT tests yields CH and CH_3 signals positive and CH_2 and C signals negative. The methyl groups from the acyl chain are centered around 18.5 ppm and the CH group from the acyl chains is centered around 39 ppm. The next characteristic signal is centered around 95 ppm, a singlet from the carbon 3. Around 103 ppm there is a slight negative signal, which corresponds the quaternary carbon between the phenol group and the acyl chain. The last two characteristic signals are centered around 172 ppm (CH carbons attached hydroxyl groups) and close to 205 ppm (carbons from the carbonyl group). When compared to Qian Yu and colleagues (Yu et al. 2016), we could find the same spectrum similarities obtained from DAPG. MAPG was previously characterized by ^{13}C NMR from Hops extract and has the same spectrum features (Bohr et al. 2005).

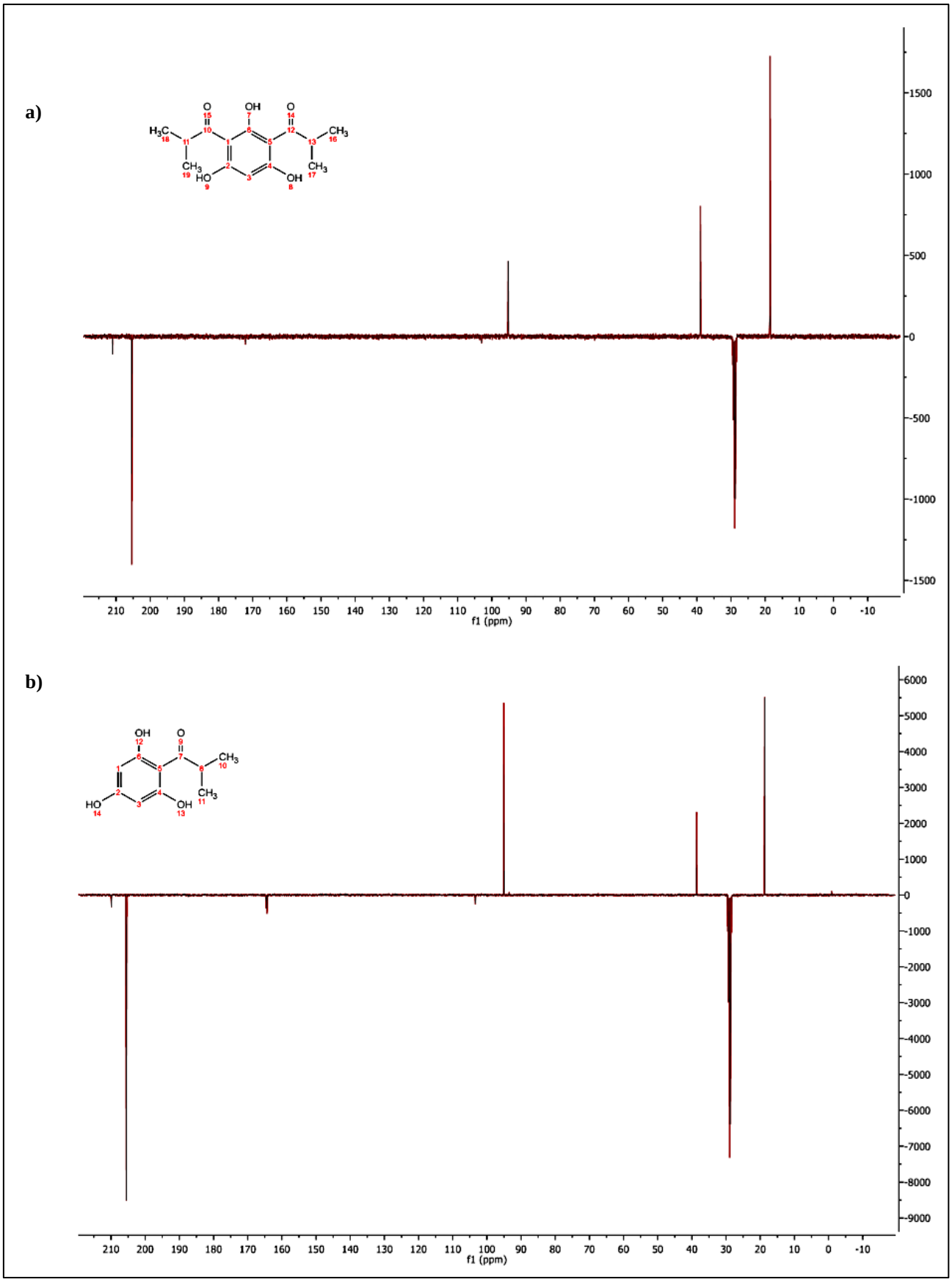


Figure 2.a) APT- ^{13}C NMR of diacylphloroglucinol (DAPG) in Acetone-d6; b) APT- ^{13}C NMR of monoacylphloroglucinol (MAPG) in Acetone-d6.

4.3 Thermal Study of DAPG and MAPG

The endothermic curves of DAPG and MAPG (Figure 3) were obtained from Diferential Scanning Calorimetry Analysis. A quick view of both figures suggests different states of purity, with DAPG showing a sharp curve around 131 ºC, corresponding to its melting point and MAPG showing multiple endothermic curves with its melting point centered around 152 ºC. Due to a greater number of ramifications, the interaction between the molecules of DAPG is weaker, having a melting point smaller than MAPG. In addition, due to its high purity, DAPG gives a sharper peak, which is characteristic of pure crystals. The endothermic curves around 80º C 220ºC from MAPG are probably derived from its degradation.

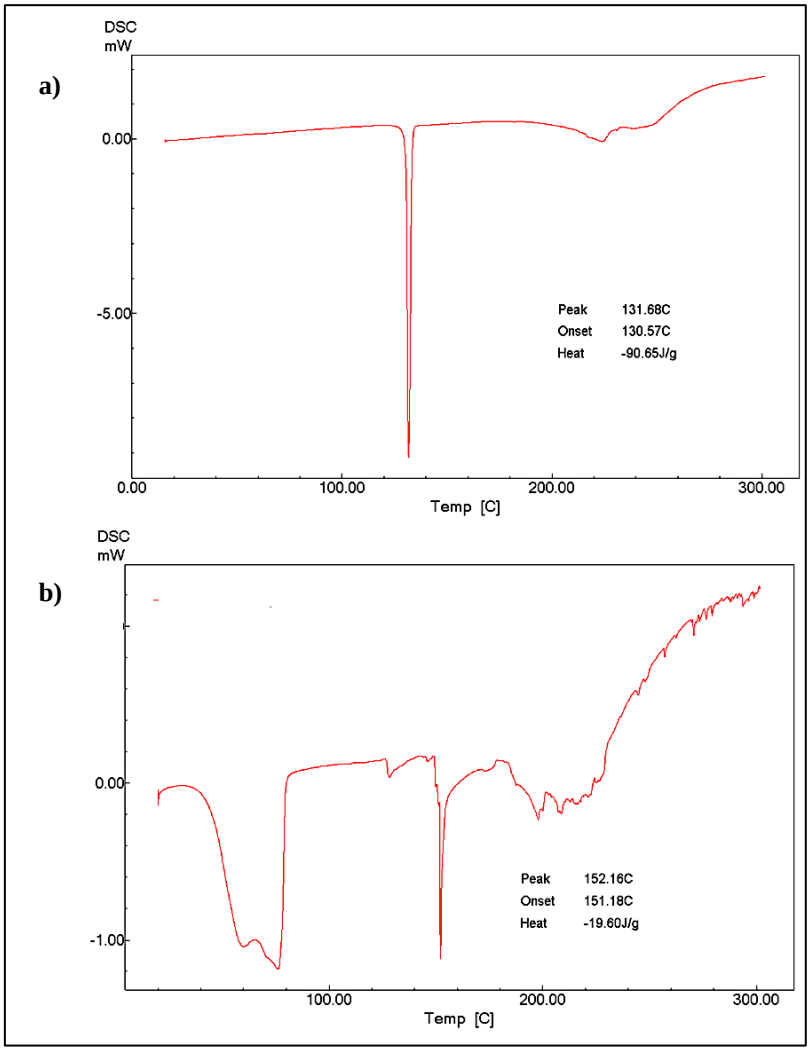


Figure 3. a) Thermogram of DAPG; b) Thermogram of MAPG

4.4 HPLC- DAD and LC-MS Analysis

To improve the characterization of DAPG and MAPG it was decided to use Diode Array Detector and Mass Spectroscopy. Both equipment give representative results and high sensitivity performances. The HPLC-DAD analysis showed a principal peak for each chromatogram, with a retention time of 5.50 and 7.65 for DAPG and MAPG, respectively (Figure 4). Due to its two acyl chains, DAPG has greater affinity to the C18 column, giving a longer retention time when compared to MAPG. The UV Spectra of DAPG and MAPG have maximum at 278 and 286 nm, respectively. Both compounds were spectroscopically pure (impurity not detect in similarity curve analysis and peak purity index greater than 0.995) (Figure 4.c and 4.f). The chromatographic purity of DAPG e MAPG was calculated by the ratio of the peak area of the major compound and the sum of all peaks areas detected in the chromatogram (total peak area). DAPG and MAPG have chromatographic purity of 95.6% and 98.0%, respectively. The results are summarized in Table 2.

Table 2. Summary of chromatographic results.

Compound	Retention time (min)	Tailing Factor	Peak Purity Index	Chromatographic Purity*
DAPG	7.65	1.17	1.0000	95.6% at 272 nm
MAPG	5.50	1.07	0.9982	98.0% at 286 nm

* Area Percent for 100 μg/mL solution of DAPG and MAPG.

LC-MS provided a simple and accurate method for identification of organic compounds. In negative mode, the DAPG spectra (Figure 4.b) showed a base peak at m/z 265.2 representative of molecular ion less one hydrogen $[M-H]^-$ and its isotopic peaks, at m/z 266.3 and 267.3, had, approximately, 15 and 2% of relative abundance, as expected for $C_{14}H_{18}O_5$. MAPG (figure 4.e) showed a base peak of 195.1 m/z, which corresponds to its molecular ion $[M-H]^-$. Isotopic peaks, at 196.2 and 197.2 m/z, were detected and its abundance are coherent with DAPG molecular formula ($C_{10}H_{12}O_4$). Therefore, the mass spectrum of DAPG showed signals at m/z 231 and 233 with a proportion of 3:1 typical of chlorine isotopes abundances. The formation of chlorine adducts are very common in negative ion electrospray ionization mass spectrometry (ESI-MS) evolving chlorinated solvents. Under appropriate conditions, chloride anions may be

produced via electrochemical reduction of chlorinated solvents at the ESI capillary, as inherent reduction processes of negative mode of ESI process (Zhu and Col 2000).

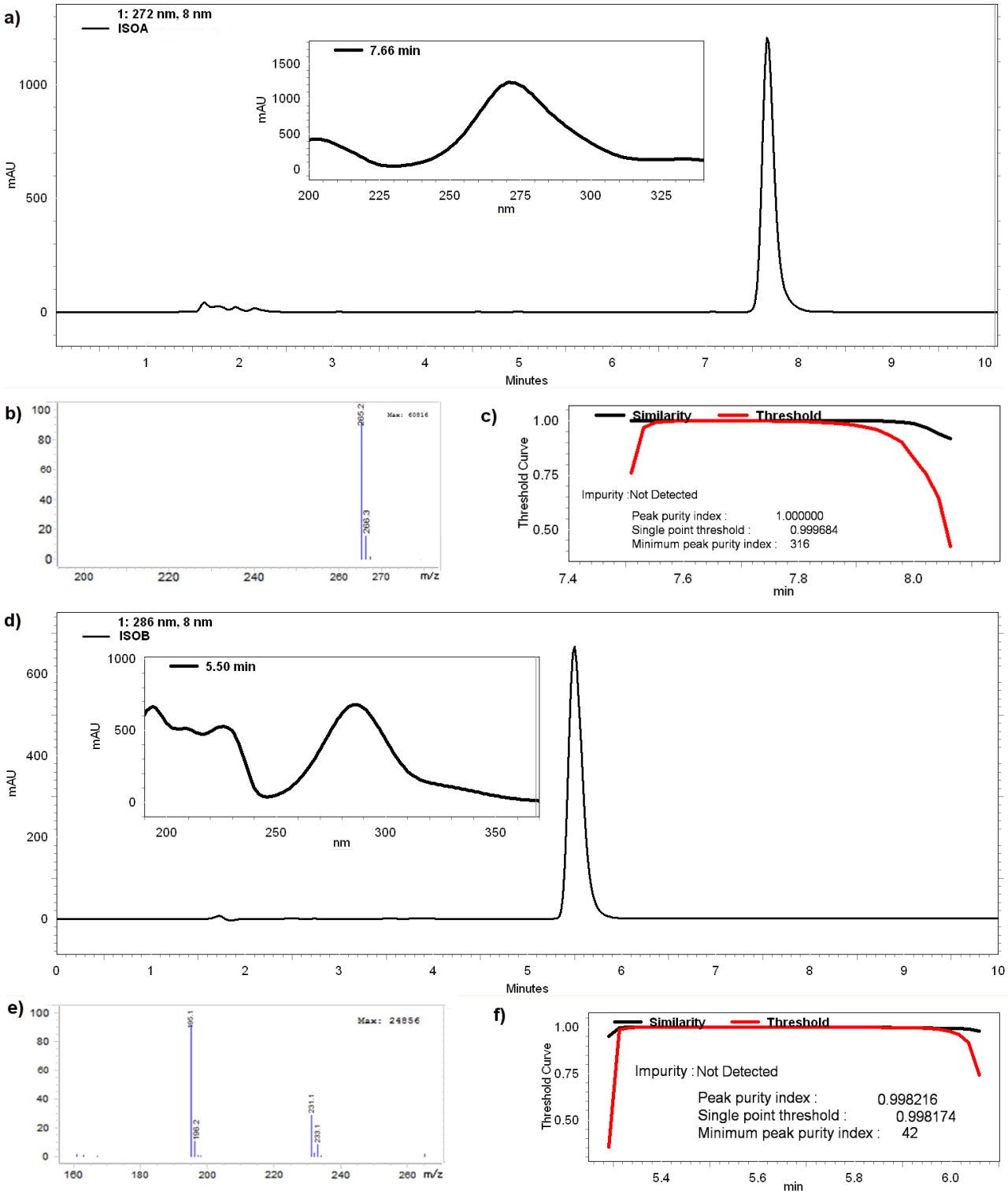


Figure 4. Representative results of HPLC-DAD and LC-MS analysis. Chromatograms of DAPG (a) and MAPG (c)

Summary of Analytical Data

DAPG- C14H18O5. 95.6% purity by HPLC-DAD.

^{1}H NMR (400 MHz, CDCl3): 1.15 (12 H, d, J= 7 Hz, 4xCH3), 3.95 (2H, m, H-9, H-10), 5.90 (1H, s, H-3).

^{13}C NMR (100 MHz, CD3COCD3): 18.5 (C-16, C-17, C-18, C-19, 4xCH3), 38.6 (C-11, C-13, 2xCH), 95.0 (C3, CH), 103.3 (C-1, C-5, Cq), 172.2 (C-2, C-4, C-6, Cq), 205.8 (C-10, C-12, CO). Bp 131 ºC. UVmax at 278 nm. MS (ESI-MS) m/z 265.2 [M-H]- and its isotopic peaks at m/z 266.3 and 267.3.

MAPG- C10H12O4. 98.0% purity by HPLC-DAD.

^{1}H NMR (400 MHz, CD3COCD3): 1.15 (6 H, d, J= 7 Hz, 2xCH3), 3.98 (1H, m, H-8), 5.90 (1H, s, H-3), 11.80 (OH).

^{13}C NMR (100 MHz, CD3COCD3): 18.7 (C-10, C-11, 2xCH3), 38.5 (C-8, CH), 95.1 (C1, C3, CH), 103.5 (C-5, Cq), 164.7 (C-2, C-4, C-6, Cq), 205.9 (C-7, CO). Bp 152 ºC. UVmax at 286 nm. MS (ESI-MS) m/z 195.1 [M-H]- and its isotopic peaks at m/z 196.2 and 197.2.

5 Conclusion

Organic molecular synthesis is a major strategy when it is desired to obtain bioactive compounds that are difficult to obtain from vegetable extraction. Phloroglucinol derivatives are molecules that have been pharmacologically tested in academic research. Therefore, the need to characterize these compounds becomes important for a structural assessment before starting the in vitro tests. We have successfully prepared and characterized two phloroglucinol derivatives using Friedel-Crafts acyaltion. The synthesis proved to be efficient and easy to prepare, giving good yields of monoacylphloroglucinol and diacylplhoroglucinol when compared to plant extraction. Furthermore, purification of DAPG by crystallization gave rise to a pure solid than MAPG, which was purified with column chromatography. These results support the fact that crystallization is a great choice to obtain products with high grade of purity, bypassing the excessive use of organic solvents and making purification faster. Characterization of DAPG and MAPG were performed by NMR, DSC, HPLC-DAD and mass spectroscopy, showing the main chemical properties of the molecules and proving the efficacy of the analysis when compared to related articles in the literature.

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