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ESI-MS, ESI-MS/MS Fingerprint and LC-ESI-MS Analysis of Proathocyanidins from *Bursera simaruba* Sarg Bark

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Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry was used to investigate the presence of proanthocyanidins (PAs) in the methanolic extract of *B. simaruba* bark. Additionally, an LC-ESI-MS qualitative study was performed by using a monolithic stationary phase. The fragmentation pattern obtained evidenced the presence in *B. simaruba* bark of PAs belonging to the series of polymers of epicatechin, along with their glycosilated derivatives.

Keywords: *Bursera simaruba* Sarg, proanthocyanidins, ESI-MS, LC-MS.

Bursera simaruba Sarg. (Burseraceae), (red gumbolimbo bark, indio desnudo), is a commonly diffused tree in Venezuela, Belize and in Central America [1]. In these countries it is traditionally used as an antidote to poisonwood sap, to treat insect bites, sunburn, rashes, skin sores, internal infections, fevers, colds and flu [2]. In addition, an interesting anti-inflammatory activity of this plant has been recently reported [3, 4]. The bark of this species is reported to be a rich source of phenolic compounds, in particular it is a good source of lignans with cytotoxic activity as yatein, β-peltatin-*O*-β-D-glucopyranoside, hinokinin and bursehernin [5]. In the present work, in the same extract, epicatechin was isolated, and identified by comparison of spectroscopic and spectrometric data with those present in literature [6]. In addition, we have evidenced the presence of proanthocyanidins, for the first time, in the methanolic extract of *B. simaruba* bark by mass spectrometry analyses.

Proanthocyanidins (condensed tannins, PAs) consisting of oligomers and polymers of flavan-3-ol units are the most widely distributed type of tannins in the plant kingdom. Dietary PAs are hypothesized to be beneficial, possibly due to their antioxidant properties and their ability to complex with macromolecules and metal ions, and they are supposed to play a role in anti-inflammatory activity of several plants [7]. Antinflammatory activity probably is related to their antioxidant and scavenging activities [8] and to their inhibition of arachidonic acid metabolism via both cyclo-oxygenase and lypoxygenase pathways [9]. Due to the complexity of PAs structures we have proposed to rationalise the presence of these compounds on the basis of ESI-MS and ESI-MS/MS profiles.

Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry was used to investigate polyphenolic compounds in the methanolic extract of *B. simaruba*. In a second stage analytical HPLC-ESI-MS and HPLC-ESI-MS/MS was developed.

PAs are very complex to separate with conventional C18 stationary phases. In our study we performed the analysis by using a monolithic C18 column, a stationary phase described to produce better resolution in different analytical fields [10]. Monoliths are rod-shaped continuous bed silica or polymeric materials, which offer an alternative to conventional particle-packed columns for analytical and preparative liquid chromatography.

ESI-MS and ESI-MS/MS fingerprint. In order to obtain qualitative information on proanthocyanidins in *B. simaruba* extract, a sample rich of these compounds was prepared and directly injected into ESI source of the mass spectrometer. Analysis of proanthocyanidins was performed in negative ion mode since it has been demonstrated for this class of

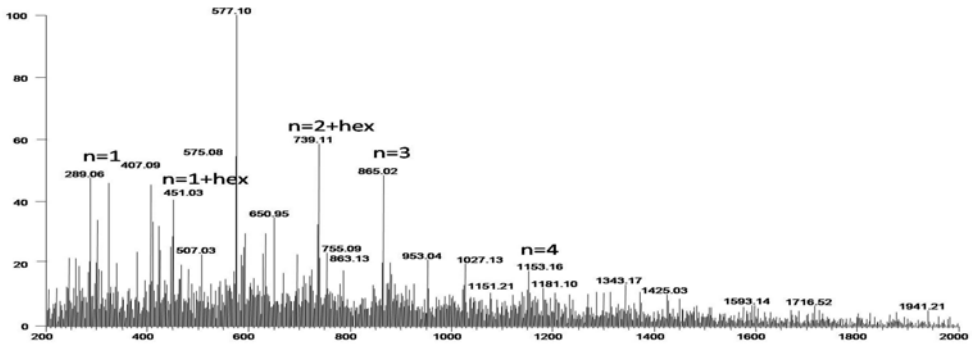

Figure 1: Typical deprotonated ESI-IT-MS fingerprint of methanolic extract of *Bursera simaruba* bark obtained in negative ion mode. n= number of epicatechin units,hex=hexose unit.

compound that negative ionization is more sensitive and selective than the positive one [11]. Figure 1 shows the ESI-MS fingerprint obtained at normal masses scan indicating the $[M-H]^-$ ions of the proanthocyanidins. The fragmentation pattern obtained evidenced the presence of glycosilated proanthocyanidins and polymeric proanthocyanidins. Together with the ion relative to epicatechin at *m/z* 289, a first series of abundant ions separated by 288 Da corresponding to ion peaks of dimeric (*m/z* 577), trimeric (*m/z* 865) and tetrameric (*m/z* 1153) procyanidins, respectively, were observed. Mass spectra also showed ions corresponding to glycosilated epicatechin (*m/z* 451) and glycosilated dimeric proanthocyanidin (*m/z* 739).

In order to verify the results described above, second-order ESI-IT-MS/MS experiments for dimeric proanthocyanidin and glycosilated dimeric proanthocyanidin were performed.

The MS^2 spectrum of the ion at *m/z* 577 (Figure 2) showed major product ions at values of 451, 425, 407 and 289 *m/z*. The ion at *m/z* 451 was derived from loss of 126 mass unit corresponding to the heterocyclic ring fission (HRF) fragmentation. The ion at *m/z* 425 $[M-152-H]^-$ was due to retro-Diels-Alder fragmentation (RDA). RDA yielded the product ion at *m/z* 407 $[M-152-18-H]^-$ due to the neutral loss of the water. Finally, the ion at *m/z* 289 was attributed to Quinone methide (QM) fragmentation $[M-288-H]^-$. This pattern of fragmentation is in accordance with those reported by Rodrigues *et al*. [11]. The product ion spectrum of the glycosilated dimeric procyanidin was also investigated. The MS^2 spectrum of the ion at *m/z* 739 (Figure 3) showed major product ions at values of 587, 577, 451, 435 and 289 *m/z*.

The ion at *m/z* 577 was derived from the neutral loss of the sugar moiety $[M-162-H]^-$ followed by the loss

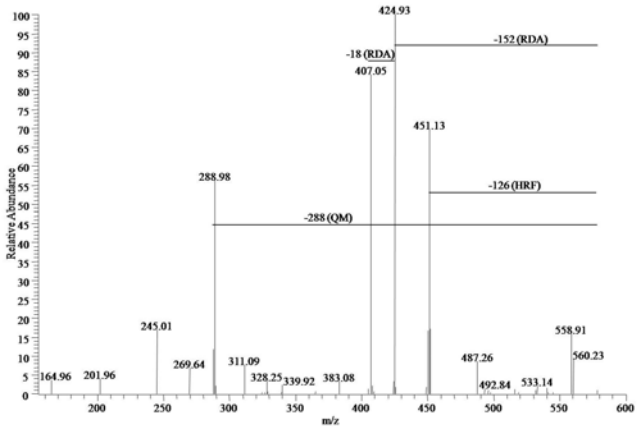

Figure 2: MS/MS spectrum of the ion at *m/z* 577.

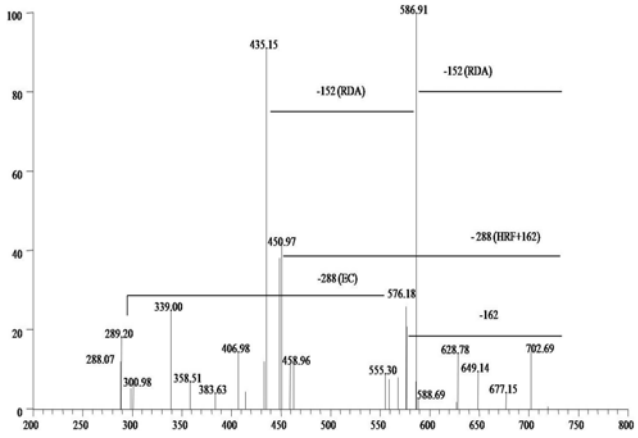

Figure 3: MS/MS spectrum of the ion at *m/z* 579.

of epicatechin unit at *m/z* 289 $[M-288-H]^-$ relative to QM fragmentation. The ions at *m/z* 587 and 435 were due to two subsequent retro Diels-Alder (RDA) fragmentations consecutive $[M-152-H]^-$ and $[M-152-152-H]^-$ respectively. Finally the ion at *m/z* 451 was derived from the loss of the ring A and the sugar unit due to HRF fragmentation. This fragmentation pattern allowed us to suppose the location of the sugar moiety only at C-5 or C-7 of ring A. Figure 4 shows the fragmentation pathway proposed for the

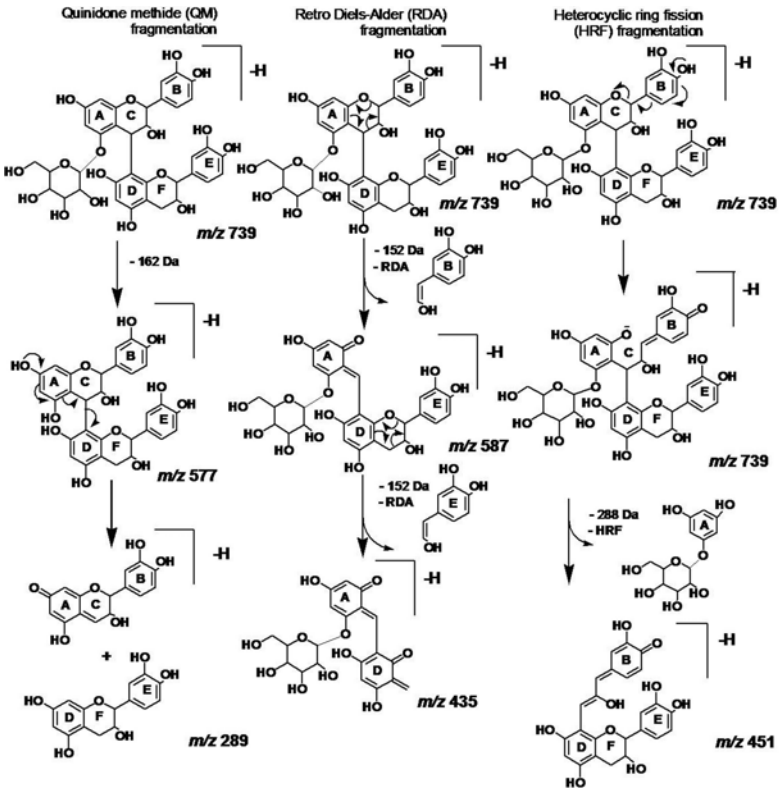

Figure 4: Main fragmentation pathways proposed for the monoglycosylated epicatechin dimer identified in the methanolic extract of *B. simaruba* barks.

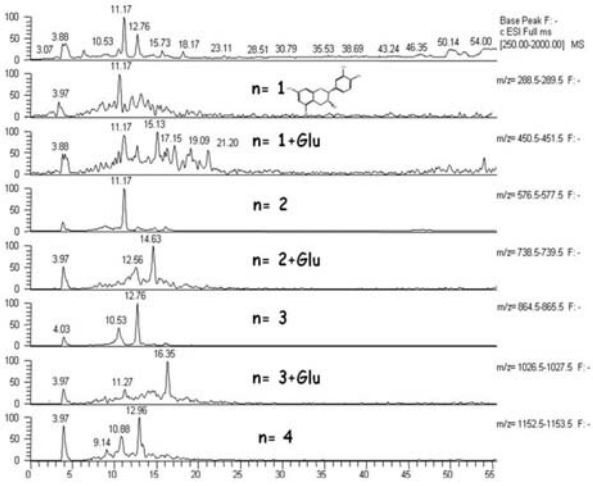

Figure 5: LC-ESI-MS RICs (Reconstructed Ion Chromatograms) of a sample enriched in proanthocyanidins from *B. simaruba* bark.

glycosilated dimeric proanthocyanidin occurring in *B. simaruba*.

LC-ESI-MS analysis: In order to realise a qualitative analysis on the epicatechin derivatives in *B. simaruba* bark methanolic extract, MS experiments were performed by using an LC-MS system equipped with an ES source and an Ion Trap analyser. Negative ion electrospray LC-ESI-MS analysis obtained in the acquisition range of 250-2000 amu (atomic mass unity), total ion current (TIC) profile and reconstructed ion chromatograms (RICs) of the methanolic extract of *B simaruba* bark are shown in Figure 5. LC-ESI-MS analysis confirmed the presence of compounds related to epicatechin.

The 250-2000 *m/z* range allowed to confirm the presence of oligomers with to four epicatechin units, but the occurence of oligomers based on a major number of epicatechins cannot be excluded. The profiles obtained for monomers appear more complex if compared to those obtained for oligomers, maybe for the occurrence of product ions generated by source fragmentation of oligomers.

Experimental

Materials: HPLC grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Merck KGaA, Darmstadt, Germany). HPLC grade water (18mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). The reagents used for the extractions, of analytical grade, were purchased from Carlo Erba (Rodano, Italy). Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Plant material: Plant material was collected in Belize in February 1999 and authenticated by

Professor M.J. Balick. Voucher specimens were dried and deposited at the New York Botanical Garden (NY, USA).

Extraction and sample preparation: The air-dried plant material (267,8 g of *B. simaruba* bark) was extracted for three days, two times, at room temperature, using solvents of increasing polarity as petroleum ether 1 L, chloroform 1 L and methanol 1 L. The solvents were removed from the filtered extracts under vacuum at 30 °C in a rotary evaporator obtaining respectively 1 g of dried petroleum ether extract; 2 g of chloroform extract and 20 g of methanol extract. Part of the methanol extract (3 g) was fractionated initially on a 100×5.0 cm Sephadex LH-20 column, using CH_3OH as mobile phase, and 120 fractions (8mL each) were obtained. Fractions grouped on the basis of their chromatographic homogeneity (preliminary MS investigation) were analyzed by ESI-MS. Fraction 32 consisted in pure epicatechin, identified by NMR and ESI-MS. A sample enriched in proanthocyanidins was prepared by combining fractions (32-120) containing proanthocyanidins.

Equipment: IT-ESI-MS, IT-ESI-MS/MS and LC-ESI-MS analysis were performed using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. Chromatography was performed on an RP C18 monolithic column Onyx.

ESI-MS and ESI-MS/MS analyses: Full scan ESI-MS and collision induced dissociation (CID) ESI-MS/MS analyses of sample were performed on a Thermo Electron (San Josè, CA, USA) LCQ Deca IT spectrometer equipped with an ion trap analyser. Sample was infused directly into the source at a flow rate of 5 μL/min. The capillary voltage was -4 V, the spray voltage was 5 kV, the capillary temperature was 270°C, sheath gas (nitrogen) flow rate 80 (arb) and auxiliary gas flow rate was 5(arb). Data were acquired in the negative ion MS and MS/MS modes.

LC-ESI-MS analysis: For qualitative LC-ESI-MS, a gradient elution was performed on an Onyx Monolithic C18 column (Phenomenex, USA), 100 x 4.6 mm, by using a mobile phase A represented by water acidified with TFA (0.05%) and a mobile phase B represented by acetonitrile acidified with TFA (0.05%). The gradient started from 0% of eluent B remained to achieve the 80% of solvent B in 55 min. The flow (250 μL min^{-1}) generated by chromatographic separation was directly injected into the electrospray ion source. The mass spectrometer was operating in the negative ion mode under the following conditions: capillary voltage -7 V, spray voltage 5 kV, tube lens offset 10 V, and capillary temperature 280°C and sheath gas (nitrogen) flow rate 80 (arb). MS spectra were acquired and elaborated using the software provided by the manufacturer.

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