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Phenolic compounds from *Bursera simaruba* Sarg. bark: Phytochemical investigation and quantitative analysis by tandem mass spectrometry

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ABSTRACT

Phytochemical investigation of the methanolic extract of *Bursera simaruba* bark led to the isolation of 11 compounds, including lignans yatein, β-peltatin-*O*-β-D-glucopyranoside, hinokinin and bursehernin, and three natural compounds namely 3,4-dimetoxyphenyl-1-*O*-β-D-(6-sulpho)-glucopyranoside, 3,4,5-trimetoxyphenyl 1-*O*-β-D-(6-sulpho)-glucopyranoside and 3,4-diidroxyphenylethanol-1-*O*-β-D-(6-sulpho)-glucopyranoside. Their structures were established by NMR and ESI/MS experiments. Additionally, an LC-ESI/MS qualitative study on the phenolic compounds and an LC-ESI/MS/MS quantitative study on the lignans found in the methanolic extract of *B. simaruba* bark were performed to give value to the plant as source of these biological active compounds. Quantitative analyses results confirmed that compounds yatein, β-peltatin-*O*-β-D-glucopyranoside, hinokinin and bursehernin are major compounds in the bark and, in particular, β-peltatin-*O*-β-D-glucopyranoside appears to be the most abundant.

1. Introduction

Bursera simaruba (Burseraceae), known as also red gumbolimbo bark or indio desnudo, is a commonly diffused tree from Venezuela, Belize and in Central America (Badillo et al., 1985). 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. Tea made from the bark is a traditional remedy for urinary tract infections (Arvigo and Balik, 1993). Another promising application for gumbolimbo bark was found in the treatment of psoriasis and eczema. In addition an interesting anti-inflammatory activity of this plant has been recently reported (Carretero et al., 2008; Noguera et al., 2004; Abad et al., 1996; Sosa et al., 2002).

Many species belonging to the genus *Bursera* have been reported to contain essential oils, triterpenes, steroids, bilignans, podophyllotoxin-like lignans and flavonoids found in particular in their resins (Culioli et al., 2003; Syamasundar and Mallavarapu, 1995), being the genus *Bursera* characterized by the presence of resins. Also for *B. simaruba* studies on the chemical composition of the resin extract are present in literature (Peraza-Sànchez and Peña-Rodrìguez, 1992; Peraza-Sànchez et al., 1995; Borges-Argaez et al., 2000). However, only one phytochemical study on metabolites produced by *B. simaruba* is present in literature which reports the isolation of the lignan yatein from the bark extract (Ciccio and Rosales, 1995). From other species of the genus *Bursera* several lignans with anti-tumor properties have been reported (Wickramaratne et al., 1995; Bianchi et al., 1968; Jolad et al., 1977a,b; Jutiviboonsuk et al., 2005; Nakanishi et al., 2005). Lignans are secondary metabolites bioactive in cancer chemotherapy and represent the bioactive constituents in a large number of medicinal plants. Many lignans are considered interesting lead structures for the development of new anti-tumoral drugs. They have been suggested to induce a wide range of biological effects, such as antioxidant, antitumor, antiviral, antibacterial, insecticidal, fungistatic, anti-platelet activities, and to protect against coronary heart disease (Kitts et al., 1999; Carletti et al., 1959; Jang et al., 2004; Borsato et al., 2000; Lima et al., 2007; Kuo et al., 2006; Markkanen et al., 1981; Wen et al., 2007; Chao et al., 2002a,b; Ichikawa et al., 1986; Chen et al., 2000; Jeong et al., 2007; Gu et al., 2002; Ikeda et al., 1998; Broomhead and Dewick, 1990a,b).

In the present study, the phytochemical composition of *B. simaruba* bark was investigated. From the methanolic extract of the bark 11 compounds (**1**–**11**) were isolated including four known lignans yatein (**1**) (Ciccio and Rosales, 1995), β-peltatin-*O*-β-D-glucopyranoside (**2**) (Rashid et al., 2000; Broomhead and Dewick, 1990a,b; von Wartburg et al., 1957), hinokinin (**3**) (Kiralj et al., 2007) and bursehernin (**4**) (Estévez-Braun et al., 1996) and seven phenolic compounds including three new compounds 3,4-dimetoxyphenyl-1-*O*-β-(6-sulpho)-glucopyranoside (**5**), 3,4,5-trimetoxyphenil 1-*O*-β-(6-sulpho)-glucopyranoside (**6**) and 3,4-diidroxyphenylethanol-1-*O*-β-D-(6-sulpho)-glucopyranoside (**7**), along with scopoletin (**8**) (Źoelek et al., 2003), phloroacetophenone 4-neohesperidoside (**9**) (Horowitz and Gentili, 1963), 4-acetyl-3,5-dihydroyphenyl-β-D-glucopyranoside (**10**) (Chosson et al., 1997) and picraquassioside D (**11**) (Yoshikawa et al., 1995). Being lignans

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1–**4** isolated from *B. simaruba* of interest for their biological activity, an high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, working in multiple reaction monitoring (MRM) mode, has been developed to quantify these compounds. The latter techniques could provide specific, selective and sensitive results for quantitative analysis with a reduced time for sample preparation if compared to other analytical techniques.

2. Results and discussion

2.1. Characterization of isolated compounds

The HRMALDI-MS analysis of compound **5** led to the molecular peak at *m/z* 395.3465 $[M-H]^-$, which indicated the molecular formula $C_{14}H_{20}O_{11}S$ (calc. for $C_{14}H_{19}O_{11}S$ 395.3591), suggesting the presence of a sulphate group in the molecule. Acid hydrolysis of **5**, followed by treatment with $BaCl_2$, gave a white precipitate, thus demonstrating the presence of a sulphate residue. The ESI-MS spectrum in negative ion mode showed the pseudo-molecular $[M-H]^-$ ion peak at *m/z* 395. The second order MS/MS spectrum of the ion at *m/z* 395 led to the significant fragment ion peaks at *m/z* 241 and *m/z* 97. The first fragmentation is due to the neutral loss of aromatic ring $[(M-H)-C_8H_{10}O_3]^-$; the second ion peak is corresponds to the sulphated group for the loss of the sugar moiety. The ^{1}H NMR spectrum of compound **5** showed signals at δ 6.90 (1H, *d*, *J* = 2.4 Hz), 6.89 (1H, *d*, *J* = 8.9 Hz) and 6.70 (1H, *dd*, *J* = 2.4, 8.9 Hz) typical of a 1,3,4 trisubstituted aromatic ring. The spectrum also showed the presence of two singlets corresponding to two methoxyl groups at δ 3.80 and 3.87. The HMBC spectrum exhibited correlations between the proton signal at δ 3.80 and the quaternary aromatic carbon at 145.6, between the proton signal at δ 3.87 and the quaternary aromatic carbon at δ 150.8, hence confirming the position of the methoxyl groups at position 3 and 4 respectively of the aromatic ring. The HSQC experiment provided all the connectivities between protons and their respective carbons as presented in Table 1. Furthermore NMR data revealed the presence of a β-glucopyranosyl unit (δ 4.76, 1H, *d*, *J* = 7.27 Hz), that was located at C-1 on the basis of the HMBC correlation between the proton signal at δ *4.76 H-1′* and the carbon resonance at δ 153.7 (C-1). The sugar unit was determined to be β-glucose. The position of the sulphate group was assigned to C-6′ on the basis of the downfield shifts of the H_2-6′ (δ4.13, *dd*, *J* = 11.3, 5.0 Hz; δ 4.44, *dd*, *J* = 11.3, 2.3Hz) and C-6′ (δ 68.2) signals, consistent with the presence of a sulphate group. On the basis of these data, compound **5** was identified as the new 3,4-dimetoxyphenyl-1-*O*-β-D-(6-sulpho)-glucopyranoside.

The HRMALDI-MS analysis of compound **6** led to the molecular ion peak at *m/z* 425.0832 $[M-H]^-$, which indicated the molecular formula $C_{15}H_{22}O_{12}S$ (calc. for $C_{15}H_{21}O_{12}S$ 425.0754), suggesting the presence of a sulphate group in the molecule. Acid hydrolysis of **6**, followed by treatment with $BaCl_2$, again demonstrated the presence of a sulphate residue. The ESI-MS of **6** showed a major ion peak at *m/z* 425 $[M-H]^-$. Its MS/MS fragmentation showed two intense peaks at *m/z* 241 $[(M-H)^- C_9H_{12}O_4]^-$ due to the neutral loss of the 3,4,5-trimethoxy phenolic ring, and at *m/z* 97 $[(M-H)^- C_6H_8O_4]^-$ due to the loss of the sugar moiety. The NMR data (1H, 13C, 2D-TOCSY, DQF-COSY, HSQC, HMBC), of **6** in comparison to those of **5** revealed that compound **6** differs from **5** only for the presence of another methoxyl group in position C-5. In fact, ^{1}H NMR spectrum showed a singlet signal at δ *6.54* correspondent to two symmetric protons (H-2, H-6), two singlets corresponding to one methoxyl group at δ 3.73 and two methoxyl groups at 3.86. The HMBC spectrum exhibited correlations between the proton signal at δ 3.73 and the quaternary aromatic carbon at 134.0, between the protons signal at δ 3.73 and the quaternary aromatic carbons at δ 154.7, hence confirming the position of the methoxyl groups at position 4 and 3, 5 respectively. Thus, the structure of compound **6** was identified as the new 3,4,5-trimetoxyphenyl-1-*O*-β-D-(6-sulpho)-glucopyranoside.

The HRMALDI-MS analysis of compound **7** led to the molecular peak at *m/z* 395.3498 $[M-H]^-$, which indicated the molecular formula $C_{14}H_{20}O_{11}S$ (calc. for $C_{14}H_{19}O_{11}S$ 395.3591), suggesting the presence of a sulphate group in the molecule. Acid hydrolysis of **7**, followed by treatment with $BaCl_2$, again demonstrated the presence of a sulphate residue. Compound **7** showed in the negative ESI-MS a major ion peak at *m/z* 395 $[M-H]^-$ and significant fragments in MS/MS analysis at *m/z* 241 and *m/z* 97.The first fragmentation is due to the neutral loss of aromatic ring $[(M-H)-C_8H_{10}O_3]^-$; the second ion peak is correspondent to the sulphated group for the loss of sugar moiety. The ^{1}H NMR spectrum of compound **7** showed signals at δ 7.20 (*s*), 6.79 (1H, *d*, *J* = 8.1 Hz) e 6.77 (1H, *d*, *J* = 8.5 Hz) typical of a 1,3,4 trisubstituted aromatic ring. The ^{1}H NMR spectrum also showed two sets of triplets (δ 2.76 and 3.75, *J* = 6.9 Hz) due to the presence of a $-CH_2-CH_2-$ unit. The HMBC spectrum exhibited correlations between the proton signal at δ 2.76 and the quaternary aromatic carbon at 132.2, hence confirming the position of the 3,4-di-

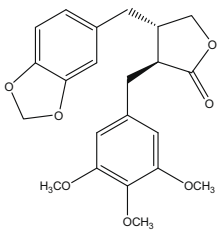
Table 1
^{13}C and ^{1}H NMR spectroscopic data of the compounds **5**, **6**, and **7** in CD_3OD.

Position	**5**		**6**		**7**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	153.7		155.7		132.4	
2	103.5	6.90 (*d*, *J* = 2.4 Hz)	95.7	6.54 *s*	119.5	7.20 *s*
3	145.6		154.7		146.4	
4	150.8		134.0		146.1	
5	113.6	6.89 (*d*, *J* = 8.9 Hz)	154.7		116.5	6.77 (*d*, *J* = 8.5 Hz)
6	108.9	6.70 (*dd*, *J* = 8.9, 2.4 Hz)	95.7	6.54 *s*	125.0	6.79 (*d*, *J* = 8.1 Hz)
7					39.2	2.76 (*t*, *J* = 6.9 Hz)
8					64.2	3.75(*t*, *J* = 6.9 Hz)
1′	103.5	4.76 (*d*, *J* = 7.3 Hz)	102.9	4.80 (*d*, *J* = 7.3 Hz)	104.1	4.76 (*d*, *J* = 7.7 Hz)
2′	74.6	3.46 (*dd*, *J* = 7.3, 9.0 Hz)	74.9	3.47 (*dd*, *J* = 7.3, 9.0 Hz)	74.6	3.53 (*dd*, *J* = 7.3, 9.0 Hz)
3′	77.5	3.47 (*dd*, *J* = 9.0, 9.0 Hz)	77.9	3.48 (*dd*, *J* = 9.0, 9.0 Hz)	77.2	3.51 (*dd*, *J* = 9.0, 9.0 Hz)
4′	71.4	3.38 (*dd*, *J* = 9.0, 9.0 Hz)	71.6	3.35 (*dd*, *J* = 9.0, 9.0 Hz)	71.1	3.42 (*dd*, *J* = 9.0, 9.0 Hz)
5′	76.0	3.69 *m*	76.2	3.74 *m*	76.0	3.71 *m*
6′	68.2	4.13 (*dd*, *J* = 11.3, 5.0 Hz)	68.5	4.09 (*dd*, *J* = 11.3, 5.0 Hz)	67.9	4.16 (*dd*, *J* = 11.3, 5.0 Hz)
		4.44 (*dd*, *J* = 11.3, 2.5 Hz)		4.49 (*dd*, *J* = 11.3, 2.3 Hz)		4.44 (*dd*, *J* = 11.3, 2.3 Hz)
3-OMe	56.7	3.80 *s*	56.5	3.86 *s*		
4-OMe	56.3	3.87 *s*	61.0	3.73 *s*		
5-OMe			56.5	3.86 *s*		

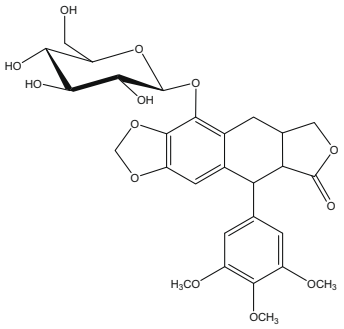
idroxyphenylethanol structure. Furthermore NMR data revealed the presence of a β-glucopyranosyl unit (δ 4.7 6, 1H, *d*, *J* = 7.7 Hz). The location of the sugar was established on the basis of the HMBC correlation between the proton signal at δ*4.76. H-1′* and the carbon resonance at δ 146.1 (C-3). The sugar unit was determined to be D-glucose and position of the sulphate group was assigned to C-6′ on the basis of the same considerations reported for compound **5**. Thus compound **7** was determined as new 3,4-diidroxyphenylethanol-1-*O*-β-D-(6-sulpho)-glucopyranoside.

^{1}H and ^{13}C NMR spectroscopic data for the compounds **5–7** are reported in Table 1.

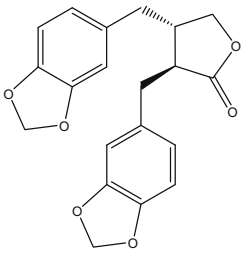
Compounds **1–4** and **8–11** have been identified by comparison of their NMR and MS data with those of the corresponding compounds reported in the literature (Ciccio and Rosales, 1995;



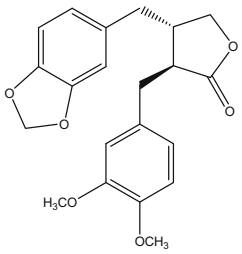
1. yatein



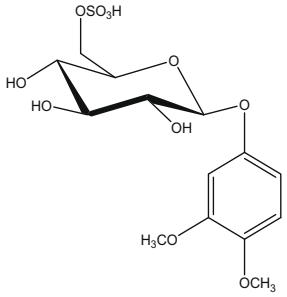
2. β-peltatin-*O*-β-D-glucopyranoside



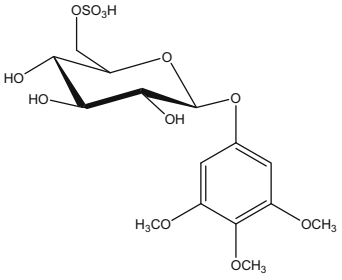
3. hinokinin



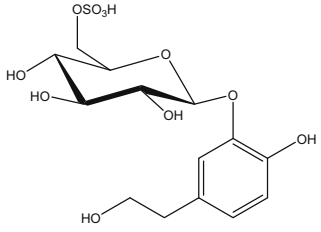
4. bursehernin



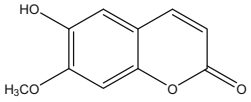
5.



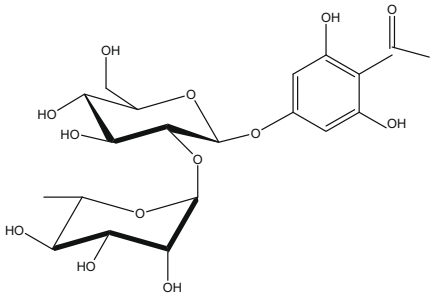
6.



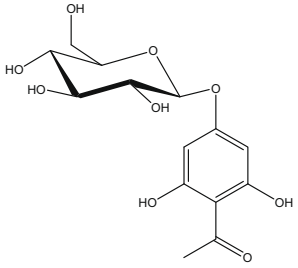
7.



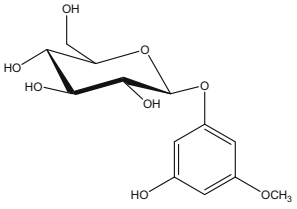
8. scopoletin



9. floroacetophenon-4-neohesperidoside



10. 4-acetil-3,5-dihydrossifenil-β-D-glucopyranoside



11. picraquassioside D

Fig. 1. Phenolic compounds from *Bursera simaruba* bark.

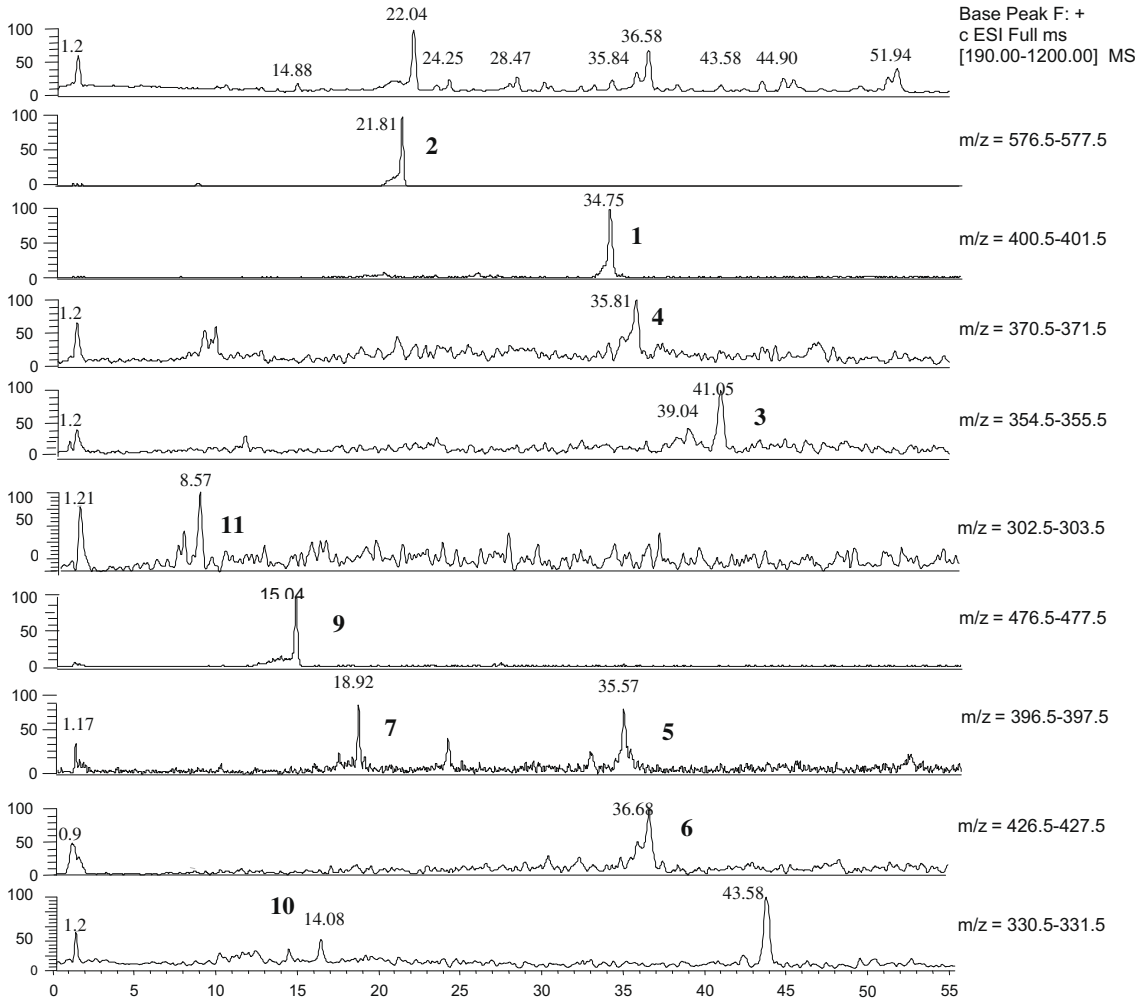


Fig. 2. LC-ESI-MS RICs (reconstructed ion chromatograms) of methanolic extract of *Bursera simaruba* bark. Chromatographic conditions: Column symmetry RP C18. Gradient: time 0 min, 100% A; time 55 min, 80% B. Compounds numbers as seen in Fig. 1.

Rashid et al., 2000; Broomhead and Dewick, 1990a,b; von Wartburg et al., 1957; Kiralj et al., 2007; Estévez-Braun et al., 1996; Źoelek et al., 2003; Horowitz and Gentili, 1963; Chosson et al., 1997; Yoshikawa et al., 1995).

All the isolated compounds are reported in Fig. 1.

Table 2
Protonated molecular ions and fragment ions of the lignans **1**–**4** acquired by FIA using ESI-MS and ESI-MS/MS.

Compound	Full MS (intensity)[a]	MS/MS (intensity)[a]
1	423 $[M+Na]^+$ (70)	401 $[M+H]^+$ parent ion (5)
	401 $[M+H]^+$ (100)	383 $[M+H-w]^+$ (20)
		365 $[M+H-2w]^+$ (5)
		181 $[M+H-220]^+$ (100)
2	599 $[M+Na]^+$ (100)	577 $[M+H]^+$ parent ion (10)
	577 $[M+H]^+$ (50)	415 $[M+H-162]^+$ (5)
		409 $[M+H-168]^+$ (20)
		247 $[M+H-330]^+$ (100)
3	377 $[M+Na]^+$ (100)	355 $[M+H]^+$ parent ion (10)
	355 $[M+H]^+$ (40)	337 $[M+H-w]^+$ (10)
		319 $[M+H-2w]^+$ (12)
		289 $[M+H-2w-30]^+$ (15)
		261 $[M+H-2w-30-28]^+$ (17)
		231 $[M+H-2w-60-28]^+$ (8)
		135 [M+H-220]+ (100)
4	393 $[M+Na]^+$ (60)	371 $[M+H]^+$ parent ion (10)
	371 $[M + H]^+$ (100)	353 $[M+H-w]^+$ (10)
		335 $[M+H-2w]^+$ (10)
		303 $[M+H-2w-32]^+$ (5)
		151 $[M+H-220]^+$ (100)

w = Water.
[a] The intensity is determined relative to the maximum mass peak.

2.2. LC-ESIMS qualitative analysis

In order to realise a qualitative analysis on the phenol derivatives in *B. simaruba* barks extracts MS experiments were performed by using an LC–MS system equipped with an ESI source and an Ion Trap analyser.

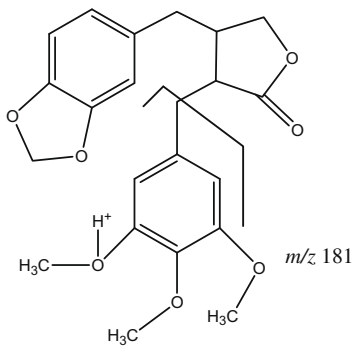


Fig. 3. Proposed major fragmentation in the positive ESI-MS/MS product ion spectrum of protonated molecular ion of compound **1** (yatein).

Positive ion electrospray LC/MS analysis obtained in the acquisition range of 190–1200 amu (atomic mass unity), total ion current (TIC) profile and reconstructed ion chromatograms (RICs), of the methanolic extract from *B. simaruba* barks are shown in Fig. 2. Phenolic derivatives were identified comparing retention times and *m/z* values in the total ion current chromatogram to those of the selected standards.

2.3. LC-ESIMS/MS lignans quantitative analysis

Due to the biological activity of lignans **1**–**4**, a quantitative LC–MS/MS method was developed. Since it is important to find a specific fragment for each analyte, ESI-MS/MS analyses were recorded for the four standard compounds by using an LC–MS instrument equipped with an ESI source and a triple quadrupole analyser

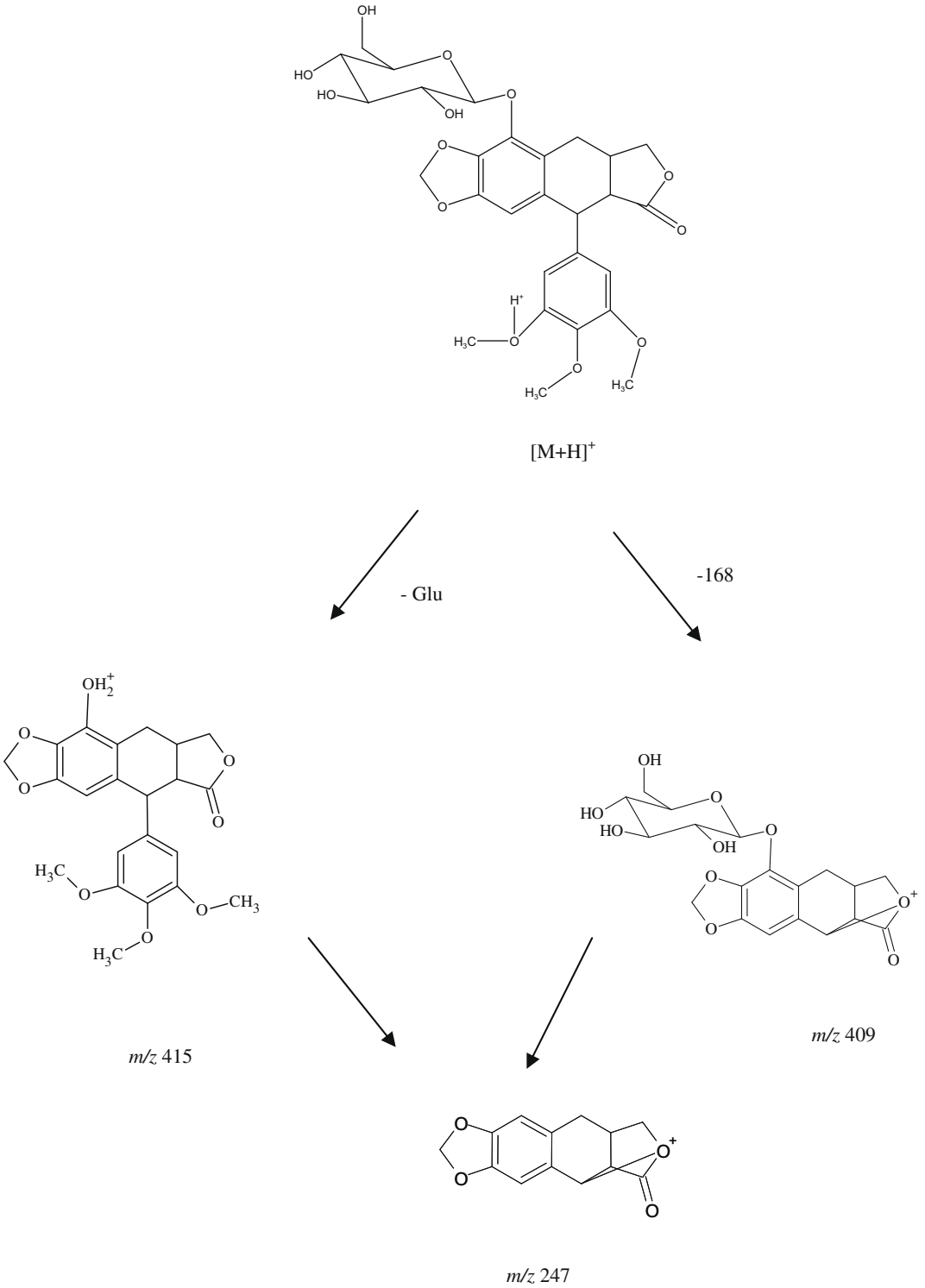


Fig. 4. Proposed structures corresponding to major product ions observed in the positive ESI-MS/MS product ion spectrum of protonated molecular ion of compound **2** (β-peltatin-*O*-β-D-glucopyranoside).

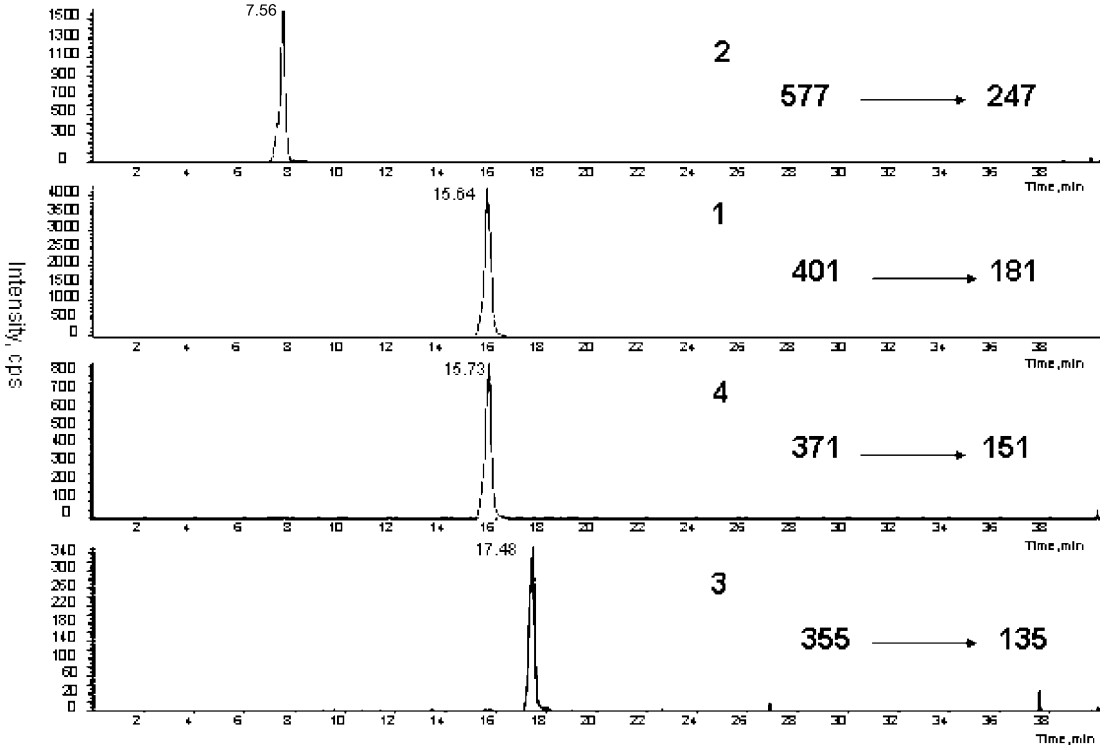


Fig. 5. LC-ES(QqQ)/MS/MS XICs (extracted ion chromatograms) of MRM analysis of compounds **1**–**4**. Chromatographic conditions: column symmetry RP C18. Gradient: time 0 min, 70% A; time 20 min, 80% B. Compounds numbers as seen in Fig. 1.

(QqQ). MS/MS Spectra results for yatein, β-peltatin-*O*-β-D-glucopyranoside, hynoikinin and bursehernin obtained by using triple quadrupole analyser are summarised in Table 2.

Compounds **1**, **3** and **4** belong to the subfamily of dibenzylbutyrolactones, while compound **2** posses the structure of an aryltetralyle lactone. Spectra of compounds **1**, **3** and **4** are characterised by a similar fragment ion corresponding to the peripheral benzene ring that retains the charge; probably it is the active site in the ESI-MS fragmentation of these compounds.

Fig. 3 reports a fragmentation and rearrangement mechanism proposed for these compounds (yatein). The mass fragment at *m/z* 181, 135 and 151 occurring in the spectra of compounds **1**, **3**, and **4** are respectively due to the charged peripheral benzene ring rearranged.

Compound **2**, β-peltatin-*O*-β-D-glucopyranoside is in the group of aryltetralin lactone lignans, in addition it is a glycosidated derivatives, thus its fragmentation is completely different.

In the ESI-QqQ-MS/MS spectrum the major fragment peaks are the ones corresponding to *m/z* 415, *m/z* 409 and *m/z* 247.

Fig. 4 shows a fragmentation and rearrangement mechanism proposed for this compound. The mass fragment at *m/z* 415, is due to the loss of the sugar moiety (glucose), while the mass fragment at *m/z* 409 is due to the loss of the peripheral benzene ring. Each of these fragments can produce a subsequent fragment at *m/z* 247, corresponding to the loss of the phenolic trisubstituted ring and sugar unit.

Thus an MRM method was developed. Transition from the specific pseudo-molecular ion of compounds **1**, **3**, and **4** to the fragment at at *m/z* 181, *m/z* 135 and *m/z* 151, and from the specific pseudomolecular ion of β-peltatin-*O*-β-D-glucopiranoside, compound **2**, to the fragment at *m/z* 247 were selected as specific reactions to monitor all the lignan derivatives.

Fig. 5 shows the MRM analyses of *B. simaruba* extract. The chromatographic profile contained all the peaks corresponding to the compounds under investigation, with appreciable intensity for quantitative purpose.

Table 3 shows quantitative analysis results; it is possible to observe that the major compound is β-peltatin-*O*-β-D-glucopiranoside, compound **2**, followed by compound **4**, burseharin, while the other compounds are present in smaller quantities. The analytical method has proven to be reliable with a good reproducibility, attested by the low values of standard deviation obtained.

2.4. Validation

Validation of the method was realised in agreement with EMEA note guidance on validation of analytical methods (ICH Q2B, 1995).

Table 3 shows calibration curve equations, regression, quantification and detection limits obtained for compounds **1–4**.

The calibration graphs, obtained by plotting area of external standard versus the known concentration of each compound, were linear in the range of 0.1–25 μg ml^{-1} for all compounds.

Detection limit was determined by the signal to noise ratio method described in the guidance, by using known low concentration of analyte and establishing the minimum concentration at which the analyte can be reliably detected. Detection limit calculated for each compound was inferior to 1.2 ng ml^{-1}.

Table 3
Quantitative results for compounds **1**–**4** by MRM LC-ESI/MS/MS.

Compound	r^2	Calibration curve equation	LOQ (ng ml^{-1})	LOD (ng ml^{-1})	Bark concentration mg kg^{-1} (D.S.)
1	0.999	$y = 7180x + 3910$	8.2	1.2	49.48 (0.02)
2	0.996	$y = 2580x - 1190$	9.9	0.9	399.44 (0.04)
3	0.999	$y = 480x + 15.1$	8.1	1.1	16.42 (0.01)
4	0.998	$y = 1180x + 505$	9.8	0.5	105.37 (0.02)

Quantification limit is defined as the lowest concentration of compound quantifiable with acceptable accuracy and precision. In the present study it was determined based on the signal to noise ratio, by injection of series of solutions until the signal to noise ratio 10 for LOQ. Each LOQ calculated for each compound was inferior to 10 ng ml^{-1}.

Compounds **1**–**4** isolated from the plant was at the purity major then 99.5%.

Validation of the LC–MS/MS method included intra and inter-day precision and accuracy studies on three days. Recoveries were determined by the addition of known quantities of the compounds under investigation to known amount of *B. simaruba* samples. Quantities were calculated by subtracting total amount of each compound before spiking to the total amount after spiking. The mean recovery of the method was 100 ± 0.6%.

3. Conclusions

B. simaruba bark is a rich source of phenolic compounds; with the exclusion of yatein, burseharin and scopoletin, all the other phenolic compounds are reported in this paper for the first time in *Bursera* genus.

The bark is a good source of lignan derivatives related to the dibenzylbutirolactone family and to the arytetralyne lactone family; some of them (yatein) are key biosynthetic intermediate of the antitumor lignan podophyllotoxin.

LC–MS/MS MRM quantitative method developed for lignan analysis was successfully applied to *B. simaruba* bark methanolic extract analysis and is also straightforward and convenient requiring a very fast sample preparation procedure; thus it is promising to be applied to other lignan producing plants to quantify these bioactive compounds in raw material and final products.

4. Experimental

4.1. Materials

HPLC grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Merck KGaA, Darmstadt, Germany). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

4.2. 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).

4.3. Preparative chromatography

Reversed-phase HPLC was carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector and a G-1365B multiple wave detector. Separations were performed on an RP C18 column μ-bondapak 300 mm × 7.6 mm (Waters, Milford, MA). Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). All solvents for chromatographic separation were of analytical grade from Carlo Erba (Rodano, Italy).

4.4. Extraction and isolation

The air-dried plant material (267.8 g of *B. simaruba* bark) were 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 cm × 5.0 cm Sephadex LH-20 column, using CH_3OH as mobile phase, and 120 fractions (8 ml each) were obtained. Fractions 16–18 (sample **a**) (136 mg), 22–23 (sample **b**) (40 mg) and 34–35 (sample **c**) (25 mg) were chromatographed by HPLC–UV using a μ-bondapak column at flow rate of 2 ml min^{-1}.

A gradient elution was performed by using a mobile phase A represented by water acidified with trifluoroacetic acid (0.05%) and a mobile phase B represented by acetonitrile acidified with trifluoroacetic acid (0.05%). From sample **a** compounds **1** (2.4 mg, t_R = 40.2), **2** (2.3 mg, t_R = 50.8) and **9** (2.6 mg, t_R = 24.7), were obtained. The elution gradient was: 0–20 min, 5–20% B, 20–35 min, 20 30% B, 35 45 min, 30–60% B and 45–55 min, 60–65% B. Sample **b** were obtained compounds **3** (0.8 mg, t_R = 58.8), **4** (1.1 mg, t_R = 56.9), **8** (2 mg, t_R = 29.9), **10** (1.7 mg, t_R = 24.1) and **11** (1.8 mg, t_R = 11.8). The elution gradient was: 0–2 min, 0–10% B, 2–12 min, isocratic elution at 10% B, 12–15 min, 10–15% B, 15–20 min, 15–20% B, 20–45 min, 20–40% B and 45–55 min, 40–60% B. From sample **c** compounds **5** (2.2 mg, t_R = 12.7), **6** (0.6 mg, t_R = 14.4) and **7** (0.9 mg, t_R = 11.5) were obtained. The elution gradient was: 0–5 min, 0–10% B, 5–10 min, 10–15% B, 10–20 min, 15–20% B, 20–25 min, 20–30% B, 25–35 min, isocratic elution at 30% B, 35–40 min, 30–35% B and 40–50 min, 35–60% B. The detection wave-length selected were 210, 254 and 350 nm.

4.5. NMR analysis

NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) at 300 K. All 2D NMR spectra were acquired in CD_3OD. The standard pulse sequence and phase cycling were used for DQF-COSY, HSQC and HMBC spectra. The NMR data were processed using UXNMR software.

All the compounds were isolated and characterized by NMR and MS comparing data with those present in the literature.

Optical rotations were measured on a JASCO DIP 1000 polarimeter.

4.5.1. 3,4-Dimetoxyphenyl 1-O-β-(6-sulpho)-glucopyranoside (*5*)

Yellow powder; $[\alpha]_D^{25}$ +26.6 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (MeOH) 254 nm, HRMALDI-MS *m/z* 395.3588 $[M-H]^-$ (calc. for $C_{14}H_{19}O_{11}S$ 395.3591). For ^1H and ^{13}C NMR spectroscopic data in CD_3OD see Table 1.

4.5.2. 3,4,5-Trimetoxyphenil 1-O-β-(6-sulpho)-glucopyranoside (*6*)

Yellow powder; $[\alpha]_D^{25}$ +20.7 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (MeOH) 254 nm, HRMALDI-MS *m/z* 425.0736 $[M-H]^-$ (calc. for $C_{15}H_{21}O_{12}S$ 425.0754). For ^1H and ^{13}C NMR spectroscopic data in CD_3OD see Table 1.

4.5.3. 3,4-Diidroxyphenylethanol-1-O-β-D-(6-sulpho)-glucopyranoside (*7*)

Yellow powder; $[\alpha]_D^{25}$ +21.9 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (MeOH) 254 nm, HRMALDI-MS *m/z* 395.3581 $[M-H]^-$, (calc. for $C_{14}H_{19}O_{11}S$ 395.3591). For ^1H and ^{13}C NMR spectroscopic data in CD_3OD see Table 1.

4.6. Preparation of phenolic standards and calibration curves

Stock solutions of standard **1–4** (l mg ml^{-1}) were prepared by dissolving each compound in MeOH. Seven different solutions, containing respectively 0.1, 0.5, 1, 2, 5, 10 and 25 μg ml^{-1} of each

compound (external standards), were prepared in MeOH and used for method development. The calibration curves, for each compound, were made by linear regression by a graph reporting the area ratio of external standard against the known concentration of external standard. The result represents the average of four curves performed by three injection of each concentration.

4.7. Sample preparation

For qualitative purpose a solution 1 mg ml^{-1} of methanolic extract was prepared and a volume of 20 μl was injected in the chromatographic system.

For quantitative purpose a solution 1 mg ml^{-1} of methanolic extract, and a volume of 10 μl was injected in the chromatographic system.

4.8. MALDI-MS, LC–MS and LC–MS/MS

Exact masses were measured by a MALDI micro MX mass spectrometer (Waters, Milford, MA). Samples were analysed by matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich Srl, Milano, Italy) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard.

Extract was analysed by LC-ESI/MS "on-line" using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. Chromatography was performed on an RP C18 column Symmetry (Waters, Milford, MA). A gradient elution was performed by using a mobile phase A represented by water acidified with trifluoroacetic acid (0.05%) and a mobile phase B represented by acetonitrile acidified with trifluoroacetic acid (0.05%). The gradient started from 0% of eluent B, to achieve the 80% of solvent B in 55 min. The flow (250 ml min^{-1}) was directly injected into the electrospray ion source. The mass spectrometer operated in the positive ion mode under the following conditions: capillary voltage 17 V, spray voltage 5 kV, tube lens offset −20 V, capillary temperature 280 °C, and sheath gas (nitrogen) flow rate 50 (arb).

LC-ESI/MS/MS experiments were carried out by using dependent scanning mode.

Standards and the solution of the methanolic extract were analysed by a 1100 HPLC system (Agilent, Palo Alto, CA) coupled with a triple quadrupole instrument API2000 (Applied Biosystems, Foster City, CA, USA). A gradient elution was performed 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 30% of eluent B to achieve the 80% of solvent B in 20 min. The flow (250 ml min^{-1}) was directly injected into the electrospray ion source.

The instrument was used in the tandem MS mode, multiple reaction monitoring (MRM). Chromatography columns used was an RP C18 Symmetry 150 × 2.1 mm (Waters, Milford, MA).

API2000 ESI source was tuned with a yatein (**1**) standard solution in methanol (l μg ml^{-1}) infused at the flow rate of 10 μl min^{-1} with a syringe pump. The mass spectrometer was operated in the positive ion mode under the following conditions: declustering potential of 32 eV, focusing potential of 400 eV, entrance potential 12 eV, collision energy 30 eV, collision cell exit potential 15 eV, ion spray voltage 5000, temperature 300 °C.

4.9. Detection of the sulphate group

A 1–2 mg aliquot of each sample **5–7** was refluxed with 10% HCl (4 ml) for 4 h and then extracted with Et_2O. An aliquot of the aqueous layer of each was treated with 70% $BaCl_2$ to give a white precipitate ($BaSO_4$).

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