Application of High Performance Liquid Chromatography Coupled with Ultraviolet Spectroscopy and Electrospray Mass Spectrometry to the Characterisation of Ellagitannins from *Terminalia macroptera* Roots

Olga Silva,[1] Elsa T. Gomes,[1,3] Jean-Luc Wolfender,[2] Andrew Marston,[2] and Kurt Hostettmann[2]

Received July 31, 2000; accepted August 8, 2000

Purpose. *Terminalia macroptera* roots are used in Guinea-Bissau and other West African countries to treat infectious diseases like gonorrhoea. Previous work showed an ethanol extract of *T. macroptera* roots (T) to have an *in vitro* antimicrobial profile against *Neisseria gonorrhoae* (including resistant strains) and enteropathogenic agents. The most active fractions of this extract were identified as the diethyl ether (T2) and water (T5) fractions. The aim of the present study was the identification of major compounds present in T and simultaneously in T2 or T5.

Methods. The T extract and T2 and T5 fractions were analysed by high performance liquid chromatography coupled with ultraviolet photodiode array (LC-UV) spectroscopy and electrospray ionization mass spectrometry (ES-MS). These analyses indicated the presence of ellagitannin derivatives. In order to confirm the identities of the detected compounds, they were isolated from T2 and T5 by preparative chromatographic techniques and identified by spectroscopic methods including tandem mass spectrometry.

Results. By using LC-UV-ES-MS, four major compounds (ellagic acid, gallic acid, punicalagin, terchebulin) could be identified in the T extract. Three other compounds (3,3′di-*O*-methylellagic acid, 3,4,3′,4′-tetra-*O*-methylellagic acid, terflavin A) were also isolated and identified.

Conclusions. LC-UV-ES-MS is a useful technique for the analysis of mixtures containing ellagitannins.

KEY WORDS: combretaceae; liquid chromatography-mass spectrometry; electrospray ionization; ellagitannins; terchebulin; *Terminalia macroptera.*

INTRODUCTION

Terminalia macroptera Guill. et Perr. is a West African species of the Combretaceae used in Guinea-Bissau by several traditional healers for the treatment of hepatitis and other infectious diseases, including venereal diseases (1). During our *in vitro* studies on biological activities of Guinea-Bissau traditional medicines, a *T. macroptera* decorticated root ethanol extract T showed a profile of antimicrobial activity against enteropathogenic micro-organisms (2) and against different strains of *Neisseria gonorrhoeae* (including strains resistant to penicillin and tetracycline) (3). Further antimicrobial studies using *Escherichia coli, Salmonella* spp., *Shigella* spp. *Vibrio cholerae* and *Campylobacter* spp. (about 100 strains of each) showed that the activity of the *T. macroptera* extract against *Campylobacter* strains was similar to that of co-trimoxazole, higher than that of sulfamethoxazole but lower than those of tetracycline, erythromycin, ampicilin and streptomycin (4).

In order to localise the biological activity, the *T. macroptera* extract T was fractionated by consecutive liquid-liquid partition and five fractions were obtained (T1 to T5). The corresponding antimicrobial activities of these fractions were determined against *Neisseria gonorrhoeae* strains and against *Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella dysenteriae, Staphylococcus aureus, Streptococcus faecalis* and *Vibrio cholerae* reference strains. Fractions T2 (diethyl ether) and T5 (water fraction) were the most active ones. Although ellagitannins have been suggested to be the major compounds (3,4), the chemical composition of *T. macroptera* root has not previously been described in detail. Recently, promising antioxidant, antiviral and antitumor activities have been reported for ellagitannins. Furthermore, this class of compounds is found widely in the food chain, in industry and in traditional herbal remedies (5).

Hyphenated techniques such as high performance liquid chromatography (HPLC) coupled to UV photodiode array detection (LC-UV) and to mass spectrometry (LC-MS) have already been demonstrated to provide useful information for the analysis of plant metabolites (6). Nevertheless, there have been few reports describing approaches employing reversed-phase HPLC interfaced to electrospray ionization (ES) for the characterisation of ellagitannins in plant extracts (7).

In this paper we report the results obtained by LC-UV-ES-MS in the analysis of the crude extract T of *T. macroptera* and in the most active fractions T2 and T5. This on-line mass spectral analysis showed the presence of a series of ellagitannins, and allowed the rapid identification of four of them. In order to confirm their structures, their isolation was undertaken from T2 and T5 by different preparative chromatographic techniques. During chromatography, three other ellagitannins were separated. The structures of the isolated compounds were elucidated by means of different spectroscopic methods, including multiple stage MS/MS (LC-ES-MS^n) experiments on an ion trap system.

ABBREVIATIONS: (TIC), total ion current chromatogram; COSY, ^1H ^1H homonuclear correlation spectroscopy; CPC, centrifugal partition chromatography; EI-MS, electron impact mass spectrometry; ES-MS, electrospray ionization mass spectrometry; HHDP, hexahydroxydiphenoyl; ^1H-^{13}C heteronuclear multiple quantum coherence; HSQC, ^1H-^{13}C heteronuclear single quantum coherence; HPLC high performance liquid chromatography; LC-TSP-MS, thermospray ionization mass spectrometry; LC-UV, high performance liquid chromatography coupled to ultraviolet photodiode array detection; MPLC, medium pressure liquid chromatography; MS^n, multiple stage mass spectrometry; NMR, nuclear magnetic resonance; NP-PEG, natural products-polyethylene glycol; R_f, retention factor; sh, shoulder; TLC, thin layer chromatography; t_R, retention time.

[1] CECF, Laboratório de Farmacognosia–Faculdade de Farmácia da Universidade de Lisboa, Av. das Forças Armadas, 1649-19 Lisboa, Portugal.

[2] Institut de Pharmacognosie et Phytochimie, Université de Lausanne, B.E.P., CH-1015 Lausanne, Switzerland.

[3] To whom correspondence should be addressed. (e-mail: etgomes@ff.ul.pt)

0724-8741/00/1100-1396$18.00/0

MATERIAL AND METHODS

HPLC-grade water was obtained by distillation employing a Büchi Fontavapor 210 distillation instrument (Flawil, Switzerland) and passed through a 0.50 μm Millipore filter (Bedford, MA, USA). HPLC-grade methanol from Maechler AG (Reinach, Switzerland) was passed through a 0.45 μm Millipore filter. Deuterated solvents for NMR experiments were purchased from Dr. Glaser AG (Basel, Switzerland). Ammonium acetate (NH_4OAc), trifluoracetic acid (TFA; spectroscopic grade), acetone and methanol (analytical grade) were obtained from Merck (Darmstadt, Germany).

Medium pressure liquid chromatography (MPLC) was carried out with a Büchi B system (Flawil, Switzerland) composed of a Büchi B-681 pump, a Büchi B-683 detector (215,254 and 366 nm) and a LKB Bromma 2210 recorder (Pharmacia LKB, Uppsala, Sweden). Fractions were collected using a Büchi 684 fraction collector and a Büchi glass column of 46 × 3.6 cm I.D. was used. A pressure of 16–20 bar was applied, giving flow rates of 5–30 ml/min.

Centrifugal partition chromatography (CPC) was performed employing a Pharma-Tech CCC-1000 apparatus (Baltimore, Maryland, USA) at a speed of 1000 rpm. A flow rate of 3 ml/min was provided by a 300 LC pump (SSI). Detection was at 254 nm using a Knauer 100 mV multi-wavelength detector (Berlin, Germany).

Semi-preparative HPLC was carried out using a Shimadzu SCL 8A preparative liquid chromatograph apparatus (Columbia, Maryland, USA). The detection was performed with a LKB Bromma 2151 variable wavelength detector, connected to a LKB Bromma 2210 recorder. Flow rates of 5 to 10 ml/min were applied.

UV spectra were recorded on-line during the HPLC analysis or on a Hitachi U2000 instrument (Tokyo, Japan).

Electron impact (EI-MS) and thermospray ionization (TSP-MS) mass spectra were obtained on a Finnigan MAT TSQ 700 (San Jose, CA, USA) triple stage quadrupole instrument.

^1H and ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR 200 instrument (Palo Alto, California, USA) at 200 and 50 MHz, respectively, or on a Varian UNITY INOVA 500 instrument at 500 and 125 MHz, respectively. Tetramethylsilane (TMS) was used as internal standard. Complete attribution of the signals was performed on the basis of 2D-experiments (^1H-^{13}C heteronuclear multiple bond coherence (HMBC) and ^1H-^{13}C heteronuclear single quantum coherence (HSQC)).

Thin layer chromatography (TLC) was performed on cellulose plates Merck (Darmstadt, Germany) employing the following solvent systems: S_1—30% aqueous acetic acid (AcOH), S_2—butanol:AcOH:water (4:1:5; upper layer) and S_3—20% aqueous AcOH. HPTLC RP18 F_{254} Merck plates were used for analytical TLC with the solvent system S_4—methanol:water (75:25). The spots were detected by UV light (366 nm) before or after spraying with the natural products-polyethylene glycol (NP-PEG) reagent (8).

Plant Material

Roots of *Terminalia macroptera* Guill. et Perr. (Combretaceae) were collected in the Contúboel region (Guinea-Bissau) and characterised by Dr. Adélia Diniz, "Centro de Botânica Tropical—IICT", Lisbon. Voucher specimen No 662 is preserved in the LISC Herbarium, Lisbon, Portugal.

Extract Preparation

After air-drying and grinding, the decorticated root (700 g) was exhaustively extracted with ethanol (80% V/V) at room temperature. The extract was concentrated under reduced pressure (<40 °C). The dried ethanol extract (T) (97.3 g) was fractionated by sequential liquid-liquid partition with n-hexane (T1), diethyl ether (T2) and water (T3). After sedimentation, the T3 fraction was filtered to give insoluble material (T4) and a water-soluble fraction (T5) (3,4).

LC-MS Samples

A solution of 20 mg of extract T diluted in 1 ml of methanol:water (1:1) was prepared. Pure compounds were analysed in a solution of 1 mg of compound in 1 ml of methanol:water (1:1). From all these solutions, 10 μl was injected onto the HPLC apparatus.

LC-UV Analysis

The analytical separations were performed on a Nova-Pak 4 μm C_{18} column (150 × 3.9 mm I.D.) from Waters (Bedford, MA, USA), equipped with a Nova-Pak Guard-Pak C_{18} pre-column. A MeOH-H_2O step-gradient was used (5:95 to 40:60 over 12 min, 40:60 to 70:30 over 8 min, 70:30 to 100:0 over 10 min). In order to avoid tailing of the phenolic compounds, 0.05% trifluoroacetic acid (TFA) was added to the solvents to give a pH of 3.0. The flow rate was 1.0 ml/min. The analysis was carried out using a Hewlett Packard 1100 series (Palo Alto, CA, USA) instrument with a photodiode array detector. The UV chromatogram was constructed at 210 and 254 nm and UV spectra were recorded between 190 and 600 nm.

LC/ES-MS Analyses

LC/ES-MS analysis was performed directly after LC/UV detection. A Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a Finnigan MAT electrospray source was used. The electrospray conditions were as follows: capillary voltage, 3.5 kV; nebulizing gas (N_2) pressure, 60 p.s.i.; stainless steel capillary temperature, 220 °C. The flow rate was kept at 1 ml/min when the extract or pure compounds were analysed by HPLC, but only 1/10 of the total eluent was divested into the source. Spectra m/z (50–2000) were recorded in the negative ion mode. LC-ES-MS^n analyses of the major ellagitannins were performed according to methodology previously described (6,9).

Isolation of Pure Compounds from Active Fractions

The T2 fraction (2.5 g) was fractionated by CPC using ethyl acetate: ligroine:methanol: water (1:1:1:1). Before injection, part of the sample precipitated (fraction 9, 600 mg), and was consequently separated. Six fractions (1 to 6) were obtained by CPC in the normal phase mode and two fractions in the reversed phase mode (7 and 8). Fraction 3 (45 mg) was subjected to semi-preparative HPLC on a LiChrospher RP-18 end-capped column, 5 μm (25 × 1.9 cm I. D., Knauer) with methanol:water (60:40) and yielded 6 (5 mg). Fraction 5 (600

mg) was subjected to gel filtration on Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO, USA; 60 × 5 cm I.D.) with methanol and yielded 1 (100 mg). Fraction 9 was submitted to MPLC on RP-18 (LiChroprep 15–25 μm, Merck) with a methanol: water gradient (25% methanol to 100%). Six fractions were obtained. Fraction 4 yielded 5 (31.4 mg) as a pure compound. Compound 7 (15 mg) was purified from fraction 5 by semi-preparative HPLC on a LiChrospher RP-18 column, 5 μm (25 × 1.9 cm I. D., Knauer) with methanol:water (60:40). Gel filtration of fraction T5 (2.9 g) on Sephadex LH-20 (57.5 cm × 4.5 cm I.D.) eluted with a gradient of methanol:water (50:50) to acetone:water (50:50) gave twenty-four fractions (1–24). Fraction 6 (76.5 mg) was subjected to semi-preparative HPLC on a LiChrospher RP-18 end-capped column, 5 μm (25 × 1.9 cm I. D., Knauer) with methanol:water (19:81) (containing 0.05% TFA) and yielded 2 (15 mg). Product 3 was obtained from fractions 14 to 16 (20 mg). Fraction 21 (56.4 mg) was subjected to semi-preparative HPLC on a LiChrosorb RP-18 column, 7 μm (25 × 2.5 cm I. D., Merck) with methanol:water (25:75) (containing 0.05% TFA), giving 4 (15 mg).

Reference Standards

Pure samples of gallic acid (1) and ellagic acid (5) were supplied by Prof. I. Fourasté (University of Paul Sabatier, Toulouse, France). Terflavin A (3) was supplied by Prof. T. Tanaka (University of Nagasaki, Japan).

RESULTS AND DISCUSSION

LC-UV

A rapid qualitative survey by LC-UV was made on the major compounds of extract T and fractions T2 and T5. LC-UV analysis of T, T2 and T5 revealed peaks with UV spectra characteristic of polyphenol derivatives, mainly of ellagitannin derivatives (2, 4, 5, 7) (Fig. 1) (10). Compounds 2 and 4 existed as pairs of isomers 2a, 2b and 4a, 4b (Fig. 1, Table 1), consistent with the presence of hydrolysable tannins with free glucose C-1 protons and which exist in different anomeric forms in solution (11).

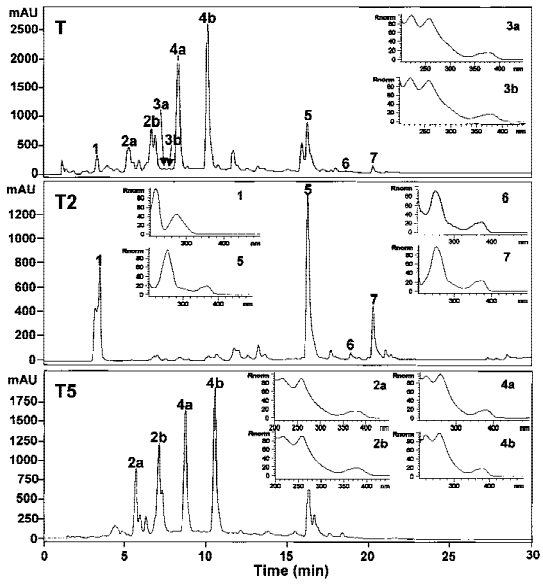

Fig. 1. LC-UV chromatograms of T extract and T2 and T5 fractions. UV chromatogram recorded at 254 nm.

In the analysed extract and fractions only the peak corresponding to compound 1 (Fig. 1, Table 1) at retention time (t_R) 3.4 min showed an on-line UV spectrum suggesting a gallic acid type of compound (10,12).

The UV spectrum of 7 (one of the T2 major compounds) (Fig. 1, Table 1) was characteristic of an alkylated ellagic acid derivative (13).

Comparison of the chromatograms for T, T2 and T5 (Fig. 1) showed that the major compounds of the extract T were recovered in both T2 and T5 fractions.

LC-UV-ES-MS

In order to obtain more information on the molecular masses of the different constituents, LC-ES-MS analyses of extract T and fractions T2 and T5 were carried out. The analyses were performed under the same conditions as for LC-UV (see *Material and Methods*). The peaks corresponding to the two major compounds (2a, 2b and 4a, 4b) recorded in the UV chromatogram (254 nm) of T gave discernible mass spectra response in the total ion current chromatogram (TIC) (Fig. 2). However, compounds 1 and 5 gave weaker mass spectra response than 2a, 2b and 4a, 4b when compared to the UV chromatogram. The TIC chromatogram of fraction T2 showed no signal for compound 7. The ES-mass spectra recorded for the same constituents in T, T2 and T5 were similar.

The ES spectra of 2a, 2b and 4a, 4b gave similar fragmentation patterns, with a deprotonated molecule $[M\text{-}H]^-$ at *m/z* 1083, together with the $[M\text{-}H\text{-}302]^-$ ion at *m/z* 781 probably due to the elimination of a hexahydroxydiphenoyl (HHDP) residue (14) (see chromatograms, Fig. 2). The obtained data indicated these compounds to be monomeric ellagitannins possessing HHDP groups (14,15). Additionally, two major peaks (p1, p2) at *m/z* 781 were detected (Fig. 2, Table 1). These peaks could be due to decomposition of the major compounds 2a, 2b and 4a, 4b. Compound 1 exhibited a $[M\text{-}H]^-$ pseudomolecular ion at *m/z* 169 indicating a MW of 170. Based on this MW and the UV spectrum compound 1 was tentatively identified as gallic acid. Compound 5 exhibited a $[M\text{-}H]^-$ ion at *m/z* 301. UV spectra and a MW of 302 suggested 5 to be ellagic acid.

The principal LC-UV-ES-MS characteristics of compounds 1, 2, 4 and 5 are summarised in Table 1.

Isolation and Identification of Compounds from Extract T

Since LC coupled to UV and ES-MS indicated the presence of several ellagitannins in extract T and could be em-

Table 1. LC-UV and ESP-MS Structural Information of 1, 2, 4, 5 and p1, p2; LC-UV Data of 7

Compound	$[M\text{-}H]^-$	UV spectra λ_{max} (nm)	t_R (min)
1	169	217, 271	3.4
2	1083	218, 258, 380	5.31, 6.69
4	1083	218, 258, 362 (sh), 381	8.29, 10.06
5	301	254, 364	16.17
7	–	249, 292 (sh), 358, 373.5	20.14
p1,p2	781	215, 256, 362 (sh), 381	5.58, 5.94

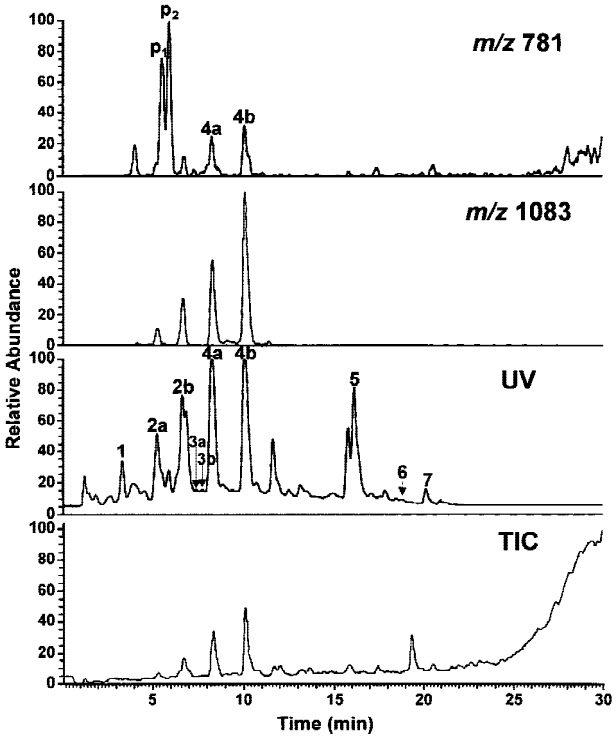

Fig. 2. LC-ESP-MS mass chromatograms of the T extract using the same chromatographic conditions as in Fig. 1. Two masses were selected: the ion chromatogram at *m/z* 1083 of the deprotonated molecule $[M-H]^-$ and the ion chromatogram at *m/z* 781.

ployed for their partial identification, their isolation was undertaken in order to complete the structure elucidation. By means of the chromatographic methods previously described, seven compounds were isolated from fractions T2 (1, 5 and 6) and T5 (2, 3 and 4). The isomers 2a, 2b and 4a, 4b could not be separated and were analysed as mixtures.

Identification of the Isolated Compounds

Compounds 1, 3 and 5 were identified by co-chromatography with authentic samples (TLC and LC-UV), respectively as gallic acid (1) (TLC: S_1 R_f 0.44; S_2 R_f 0.54 (blue-violet). LC-UV see Table 1), α,β-terflavin A (3) (TLC: S_1 R_f 0.43; S_3 R_f 0.34 (olive green with NP-PEG reagent). LC-UV: t_R 6.88, 8.27 min., on-line UV λ_{max}: 221.5, 260, 375 nm) and ellagic acid (5) (TLC: S_1 R_f 0.06; S_2 R_f 0.2 (light blue). LC-UV, see Table 1). Terflavin A (3) is a minor constituent of T and T5.

The mixture of compounds 2a and 2b was identified by comparison of their ^{1}H-, ^{13}C- and 2D NMR data with those reported in the literature for α,β-punicalagin (16–18). The ES-MS generated deprotonated molecule $[M-H]^-$ at *m/z* 1083 (1084 MW) and UV spectra exhibited by the isomers of 2 confirmed this identification.

The ^{1}H- and ^{13}C-NMR data of the mixture of compounds 4a and 4b (main compounds present in *T. macroptera* root) were similar to those reported in the literature for the anomeric mixture of α,β-terchebulin (18,19). The $[M-H]^-$ ion at *m/z* 1083 was in agreement with this identification. In order to obtain further confirmation, an ES-MS^n experiment was also performed on this mixture (Fig. 3). The $[M-H]^-$ ion at 1083 (Fig. 3a) yielded a fragment ion at *m/z* 1065 $[M-18-H]^-$ corresponding to the loss of a molecule of water ($[M-H-H_2O]^-$) and a second fragment ion at *m/z* 781 corresponding to the loss of a HHDP residue (Fig. 3b). The MS^2 spectrum of *m/z* 781 first generation fragment ion showed two major product ions, one at *m/z* 601 corresponding to the combined loss of water, a glucose residue and a HHDP residue ($[M-H_2O$-Glu-HHDP$]^-$) and a second at *m/z* 449. The later ion was also present in the MS^4 spectrum of the *m/z* 601 ion (Fig. 3c), indicating the further loss of galloyl residues (15). These results are in accordance with the proposed identification.

Compound 6 is a minor constituent of T and T2 and was identified as 3,3′-di-*O*-methylellagic acid by comparison of the spectral data obtained with data from the literature (13,20), particularly by analysis of the UV spectral data

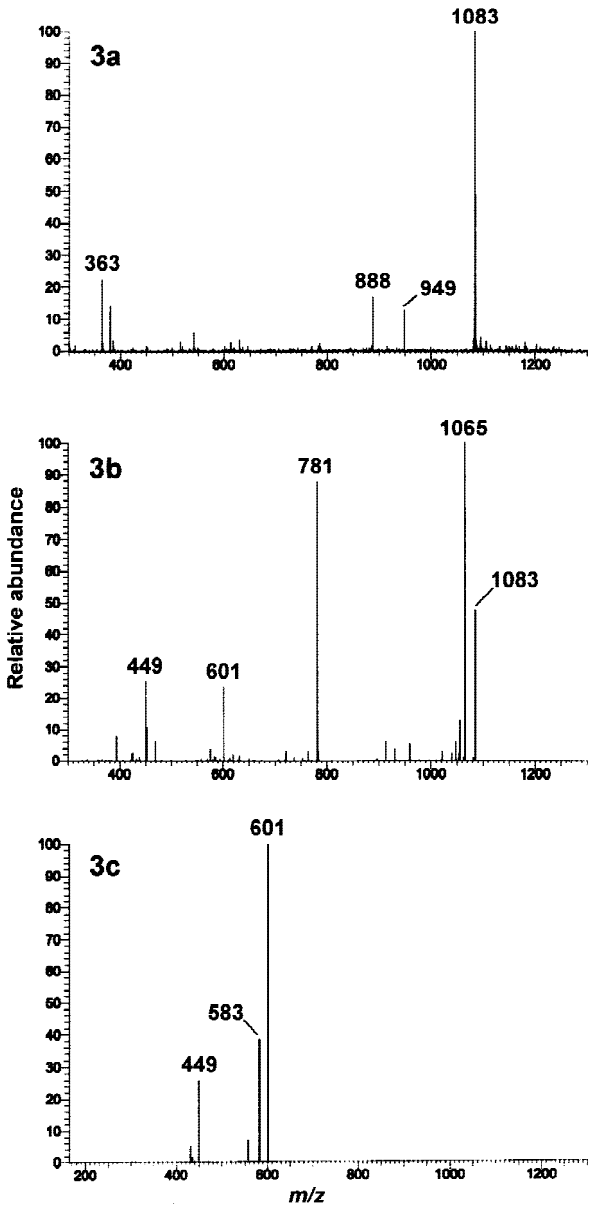

Fig. 3. ES and ES-MS^n spectra of 4a and 4b; Fig. 3a: first generation ES spectrum; Fig. 3b: MS^2 product ion spectrum of the *m/z* 1083 ion; Fig. 3c: MS^4 product ion of the *m/z* 601 generated according to the sequence *m/z* 1083 – *m/z* 781 – *m/z* 601.

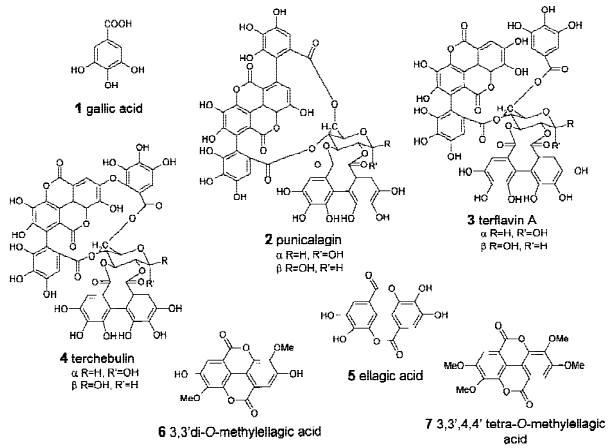

Fig. 4. *Terminalia macroptera* roots isolated compounds: gallic acid (1), punicalagin (2), terflavin A (3), terchebulin (4), ellagic acid (5), 3,3′-di-*O*-methylellagic acid (6) and 3,3′,4,4′-tetra-*O*-methylellagic acid (7).

recorded before and after addition of sodium acetate. As reported by Sato (13) it is possible to distinguish between 3,3′- and 4,4′-di-*O*-methylellagic acids by their different UV absorption patterns and bathochromic shifts in the long wavelength band after the addition of alkali; only the 3,3′-di-*O*-methyl derivative shows bathochromic shifts greater than 10 nm. HSQC correlations observed between C-5 and H-5 and C-5′ and H-5′ also confirmed this identification. Physical and chemical characteristics of 6, 3,3′-di-*O*-methylellagic acid: $C_{14}H_6O_8$. Yellow powder. TLC: S_2, R_f 0.71; S_4 R_f 0.33 (blue). HPLC: t_R 16.28 min. UV λ_{max} (MeOH): 248, 288 (sh), 355, 375; (MeOH + NaOAc (1 mg)): 256, 302 (sh), 366, 406 nm. TSP-MS (*m/z,* rel. int.):331 $[M + H]^+$ (100).

^{1}H NMR (500 MHz, CD_3COCD_3-D_2O): δ 7.64 (2H, s, H-5, H-5′), 4.19 (6H, s, OCH_3-3, OCH_3-3′). ^{13}C NMR (125 MHz, CD_3COCD_3-D_2O), HSQC: δ (selected) 112.4 (C-5(5′)), 61.64 ($O\underline{C}H_3$).

Compound 7 is a minor constituent of T but a major constituent of T2. The chemical and UV spectral data of this compound were found to be very similar to those reported for ellagic acid derivatives (13). The mass spectrum showed a $[M]^+$ at *m/z* 358 and loss of 15 mass units, a fragmentation pattern which is in agreement with that reported for methylated ellagic acid derivatives (20), more specifically, tetramethylated ellagic acid. A study of the ^{1}H NMR and ^{13}C NMR of 7 lent further support to this proposal. The symmetrical molecule 7 gave a ^{1}H NMR spectrum which contained only 3 singlets (s): one at δ 7.49 (due to the two identical protons at C-5 and C-5′; one at δ 4.0 attributed to the two methoxy groups present at C-3 and C-3′ and one at δ 3.9 assigned to the methoxy groups present at C-4 and C-4′. The ^{13}C NMR spectrum of 7 showed 9 signals: one signal at δ 159.7 for the carbonyl carbons, three signals in the region from δ 140 to 152 for the oxygenated carbons, three signals in the region from δ 106.7 to 115.4 for the protonated and quaternary carbons and two signals at δ 61.1 and 56.4 for the methoxy groups. The correct signal attribution was confirmed by gradient HMBC and HSQC experiments. Accordingly, compound 7 was identified as 3,3′,4,4′-tetra-*O*-methylellagic acid (21), the spectral data of which have not previously been published. Physical and chemical characteristics of 7, 3,3′,4,4′-tetra-*O*-methylellagic acid: $C_{18}H_{14}O_8$. Yellow needles. TLC: S_2, R_f 0.83; S_4 R_f 0.24 (blue). HPLC: t_R 20.14 min. UV λ_{max} (MeOH): 249, 292 (sh) 358, 373.5 nm. EI-MS (*m/z,* rel. int.): 358 $[M]^+$ (100), 343 $[M\text{-}15]^+$ (18.2), 300 $[M\text{-}15\text{-}CH_3CO]^+$ (11.3), 272 (7.2), 215 (7.6), 201 (10.5), 145 (13.7), 117 (13.3), 91 (9.0), 74 (10.5), 55 (10.6), 44 (30.4). ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.49 (2H, s, H-5, H-5′), 4.0 (6H, s, OCH_3-3, OCH_3-3′), 3.9 (6H, s, OCH_3-4, OCH_3-4′). ^{13}C NMR (125 MHz, CD_3COCD_3): δ 159.7 (C-7(7′)), 152 (C-4(4′)), 142.8 (C-2(2′)), 140 (C-3(3′)), 115.4 (C-6(6′)), 109.2 (C-1(1′)), 61.1 ($O\underline{C}H_3$-C(3), $O\underline{C}H_3$-C(3′)), 56.4 ($O\underline{C}H_3$-C(4), $O\underline{C}H_3$-C(4′)).

CONCLUSION

A rapid identification of the main compound terchebulin (4) and of the biogenetically related tannins punicalagin (2), gallic acid (1) and ellagic acid (5) in the extract T from *Terminalia macroptera* roots was performed by LC-UV-ES-MS (Fig. 4). Subsequently, terchebulin (4), punicalagin (2) and terflavin A (3) (18) were isolated from a *Terminalia macroptera* root extract. All these compounds are ellagitannins with hexahydroxydiphenoyl groups esterifying the hydroxyl groups of one glucose core (5).

Gallic acid (1), ellagic acid (5) and two alkylated ellagic acid derivatives (6 and 7) were also isolated. Although 3,3′,4,4′-tetra-*O*-methylellagic acid (7) has been isolated from other sources (21), the corresponding spectral data presented are, to our knowledge, reported for the first time here.

This work also proves the value of the negative ion ES-MS technique for the molecular weight determination of ellagitannins in their free forms. Previously, mass spectra of punicalagin and terchebulin were obtained by FAB-MS in the negative ion mode (15,19). Combined LC-UV-ES-MS data provided useful structural information for this class of compounds. MS^n experiments were also found to be very helpful for the determination of the structure of terchebulin. Determination of the antibacterial activity of compounds 1 to 7 is now in progress.

ACKNOWLEDGMENTS

The first author gratefully acknowledges the Portuguese Ministry of Science and Technology for a Ph.D. fellowship (Praxis XXI/BD/11429/97). Financial support was provided by Swiss National Science Foundation and by the Portuguese Ministry of Science and Technology.

REFERENCES

1. M. A. Diniz, O. Silva, M. A. Paulo, and E. T. Gomes. Medicinal uses of plants from Guinea-Bissau. In L. J. G. van der Maesen (eds.) *The Biodiversity of African Plants,* Kluwer Academic Publishers, Dordrecht, 1996, pp. 727–731.
2. O. Silva, A. Duarte, J. Cabrita, M. Pimentel, A. Diniz, and E. T. Gomes. Antimicrobial activity of Guinea-Bissau traditional remedies. *J. Ethnopharmacol.* **50**:55–59 (1996).
3. O. Silva, E. Ferreira, M. V. Vaz Pato, and E. T. Gomes. Guinea-Bissau's Plants *In vitro* susceptibility studies on *Neisseria gonorrhoeae. Int. J. Pharmacog.* **53**:323–328 (1997).
4. O. Silva, A. Duarte, M. Pimentel, S. Viegas, H. Barroso, J. Machado, I. Pires, J. Cabrita, and E. T. Gomes. Antimicrobial activity of *Terminalia macroptera. J. Ethnopharmacol.* **57**:203–207 (1997).
5. R. G. S. Berlinck, T. Hatano, T. Okuda, and T. Yoshida. *Progress in the Chemistry of Organic Natural Products 66,* Springer-Verlag, Wien, 1995.

6. J.-L. Wolfender, S. Rodriguez, and K. Hostettmann. Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. *J. Chromatogr. A.* **194**:299–316 (1998).
7. M. A. M. Nawwar, M. S. Marzouk, W. Nigge, and M. Linscheid. High-performance liquid chromatographic electrospray ionization mass spectrometric screening for polyphenolic compounds of *Epilobium hirsutum*—The structure of the unique ellagitannin epilobamide-A. *J. Mass Spectrom.* **32**:645–654 (1997).
8. H. Wagner. *Plant Drug Analysis,* Springer-Verlag, Heidelberg, 1984, pp. 299–304.
9. C. Perret, J.-L. Wolfender, and K. Hostettmann. LC/ES-MS analysis of triterpene glycosides: Rapid estimation of the saponin content of *Phytolacca dodecandra* dried berries. *Phytochem. Anal.* **10**:272–278 (1999).
10. E. Cadahía, E. Conde, M. C. García-Vallejo, and B. Fernández de Simón. High pressure liquid chromatographic analysis of polyphenols in leaves of *Eucalyptus camaldulensis, E. globulus* and *E. rudis:* Proanthocyanidins, ellagitannins and flavonol glycosides. *Phytochem. Analysis* **8**:78–83 (1997).
11. T. Hatano, T. Yoshida, and T. Okuda. III. Multiple peaks in high-performance liquid chromatography of some hydrolyzable tannins. *J. Chromatogr. A.* **435**:285–295 (1988).
12. C. F. Van Sumere. Phenols and phenolic acids. In J. B. Harborne (eds.), *Methods in Plant Biochemistry: Plant Phenolics,* Academic Press Limited, London, 1989, p. 63.
13. T. Sato. Spectral differentiation of 3,3′-di-*O*-methylellagic acid from 4,4′-di-*O*-methylellagic acid. *Phytochemistry* **26**:2124–2125 (1987).
14. R. Self, J. Eagles, G. C. Galletti, I. Mueller-Harvey, R. D. Hartley, A. G. H. Lea, D. Magnolato, U. Richli, R. Gujer, and E. Haslam. Fast atom bombardment mass spectrometry of polyphenols (*syn.* vegetable tannins). *Biomed. Environ. Mass Spectrom.* **13**:449–468 (1986).
15. R. Isobe, T. Tanaka, G. Nonaka, and I. Nishioka. A new method for structural study of hydrolyzable tannins by negative ion fast atom bombardment mass spectrometry. *Chem. Pharm. Bull.* **37**: 1748–1750 (1989).
16. A. J. Doig, D. H. Williams, P. B. Oelrichrs, and L. Baczynskyj. Isolation and structure elucidation of punicalagin, a toxic hydrolysable tannin, from *Terminalia oblongata. J. Chem. Soc. Perkin Trans. I* :2317–2321 (1990).
17. A. Jossang, J.-L. Pousset, and B. Bodo. Combreglutinin, a hydrolyzable tannin from *Combretum glutinosum. J. Nat. Prod.* **57**:732–737 (1994).
18. T.-C. Lin, Y.-T Ma, F.-L. Hsu. Tannins from the bark of *Terminalia arjuna. Chin. Pharm. J.* **48**:25–35 (1996).
19. T.-C. Lin, G. Nonaka, I.-I. Nishioka, and F.-C. Ho. Tannins and related compounds. CII. Structures of terchebulin, an ellagitannin having a novel tetraphenylcarboxylic acid (terchebulic acid) moiety, and biogenetically related tannins from *Terminalia chebula* Retz. *Chem. Pharm. Bull.* **38**:3004–3008 (1990).
20. M. A. M. Nawwar, J. Buddrus, and H. Bauer. Dimeric phenolic constituents from the root of *Tamarix nilotica. Phytochemistry* **21**:1755–1758 (1982).
21. Chapman & Hall. Ellagic acid, derivative: Tetra-Me ether, In *Dictionary of Natural Products on CD-ROM,* release 6:1, Chapman & Hall, London, 1997.