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Catechin derivatives in *Jatropha macrantha* stems: Characterisation and LC/ESI/MS/MS quali–quantitative analysis

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Abstract

A phytochemical investigation on methanol extract of stems of *Jatropha macrantha* led to the isolation of catechin, catechin-7-*O*-β-glucopyranoside and proanthocyanidin B-3 along with other catechin polymers. Their structures were established by NMR and ESI/MS experiments. Additionally, an LC/ESI/MS qualitative study and an LC/ESI/MS/MS quantitative study of the phenolic fraction of *J. macrantha* stems were performed. Combination of high performance liquid chromatography (HPLC) (DAD) with positive electrospray ionisation (ESI) and tandem mass spectrometry (MS/MS) performed with Ion Trap analyser permitted to have qualitative data on catechin derivatives: several other proanthocyanidins were detected. A mixture of proanthocyanidin polymers was characterised by direct introduction ESI-MS analysis. An LC/ESI/MS/MS method was developed and validated for separation and quantification of catechin, catechin-7-*O*-β-glucopyranoside and proanthocyanidin B-3. Due to the sensitivity and the repeatability of the assay, we suggest this method as suitable for industrial quality control of raw materials and final products. Quantitative analyses results confirmed that compounds **1**–**3** are major compounds of the plant and, in particular, proanthocyanidin B-3 appears to be the most abundant.

Keywords: *Jatropha macrantha*; Catechin; LC/ESI/MS; Proanthocyanidins

1. Introduction

"Huanarpo macho" [*Jatropha macrantha* Müll. Arg; Euphorbiaceae] is a small shrub that grows on the Peruvian coast and in the highlands. Traditionally the stems, when prepared as tinctures or aqueous infusions, have been used both as a potent aphrodisiac as well as a general energizer [1,2]. Recently this plant has shown an upsurge on the international phytotherapeutic market as sexual stimulant and energizer associated either with *Lepidium meyenii* Walp (Cruciferae) or with *Ptychopetalum olacoides* Benth (Olacaceae). Previous pharmacological studies reported the use of this plant to increase sex hormone levels, to treat menopause disorders and as lipase inhibitors [3–5]. A previous phytochemical study reports the occurrence of alkaloids; however we could not confirm this result [6].

Our phytochemical investigation on methanol extract of stem of *J. macrantha* led to the isolation of catechin, catechin-7-*O*-β-glucopyranoside and proanthocyanidin B-3 along with other catechin polymers. Several authors reported in the last years the possible role of proanthocyanidins (condensed tannins) as sexual stimulant, specifically able to correct erectile dysfunctions and infertility [7–10]. Thus, the high amount of catechin derivatives in *J. macrantha* is in agreement with the traditional use of this plant as an aphrodisiac.

Proanthocyanidins (condensed tannins) consisting of oligomers and polymers of flavan-3-ol units are the most widely distributed type of tannins in the plant kingdom [11]. Humans consume significant quantities of proanthocyanidins in foods, such as fruits, vegetables, cereals, legumes, and grains, as well as in beverages, including tea, cocoa, and red wine [12]. Dietary proanthocyanidins are hypothesized to be beneficial, possibly due to their antioxidant properties and their ability to complex with macromolecules and metal ions [13]. In nature, these compounds with their anti-feedant activities due to the ability of complex and precipitate proteins can influence the behavior of plant-feeding insects and mammals [14,15].

Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are among the most powerful techniques for the elucidation of phenolic structure. During the past decade, electrospray ionisation (ESI) MS has emerged as highly useful

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methodology for the direct conjugation with liquid phase separation techniques such as chromatography [16] and electrophoresis [17]. The utility of high performance liquid chromatography (HPLC) separation was greatly enhanced by mass spectrometric detection, which allowed to confidently identify the compounds in plant materials [18]. Liquid chromatography coupled to mass spectrometry (LC/MS) can give information on sugar and acyl moieties in phenolic compounds not revealed by the UV spectrum without the need to isolate the compounds [19].

For quantitative purpose in the last decade LC/ESI-MS/MS has become one of the major tools for biological and chemical analyses [20]. LC–MS/MS techniques frequently provide specific, selective and sensitive quantitative results often with reduced sample preparation and analysis time if compared to other commonly employed techniques.

2. Materials and methods

2.1. Materials

Epigallocatechin, used as internal standard, was purchased from Sigma Chemical Co. (St. Louis, MO). Standards of pure compounds **1**–**3** were isolated in our laboratory and their structures were elucidated by NMR (Fig. 1). HPLC grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

2.2. Plant material

J. macrantha Müll. Arg (Euphorbiaceae) stems were collected in Puerto Maldonado, Peru, in May 2004. Herbarium voucher specimens were prepared, identified and deposited at the Herbarium of the Museum of Natural History of the Universidad Nacional Mayor de San Marcos (Lima, Peru).

2.3. Preparative chromatography

HPLC separations were carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector, a G-1322A degasser, and a G-1315A photodiode array detector using a 30 cm × 7.6 mm i.d. μ-Bondapack RP-18 column (Waters Corp., 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).

2.4. Extraction and isolation procedures

Dried and powdered stems (400 g) of *J. macrantha* were extracted for a week, three times, at room temperature using solvents of increasing polarity, namely, petroleum ether 1.5 L, chloroform 1.5 L, and methanol 1.5 L, to afford 0.5, 9.98, and 60.11 g, respectively. The extractive solutions were dried in a rotavapor at 40 °C. 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 80 fractions (8 mL each) were obtained. Fractions 28–33 (49.8 mg) and 34–41 (58.5 mg) were chromatographed by HPLC on a 300 mm × 7.6 mm i.d. μ-Bondapack RP-18 (Waters) column using CH_3OH/H_2O (1:4) as eluent flow rate of 2.5 mL min^{-1} to afford compound **2** (12 mg, t_R = 13 min) (catechin-7-*O*-β-D-glucopyranoside, Fig. 1) from the fractions 28–33, and compound **1** (15.9 mg, t_R = 31 min) (catechin, Fig. 1) from the fractions 34–41.

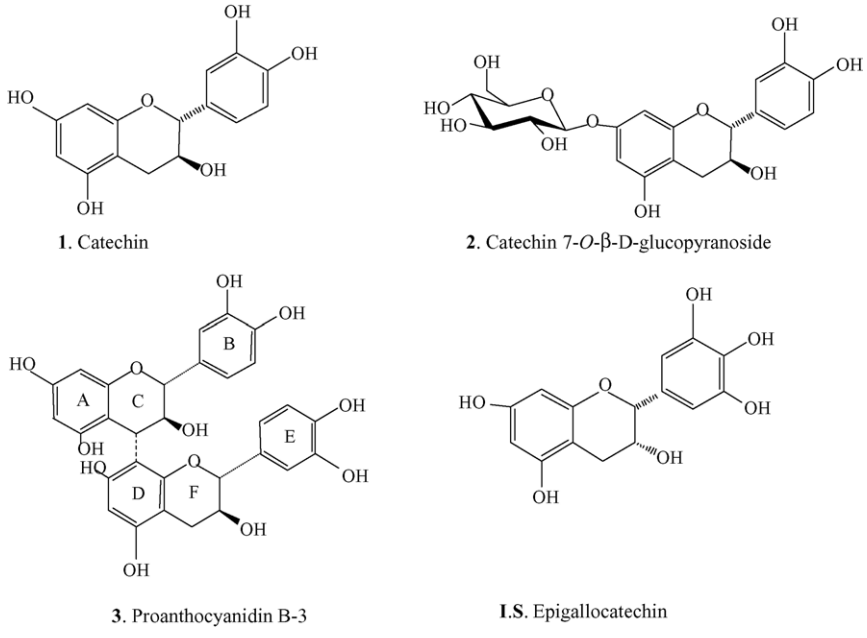
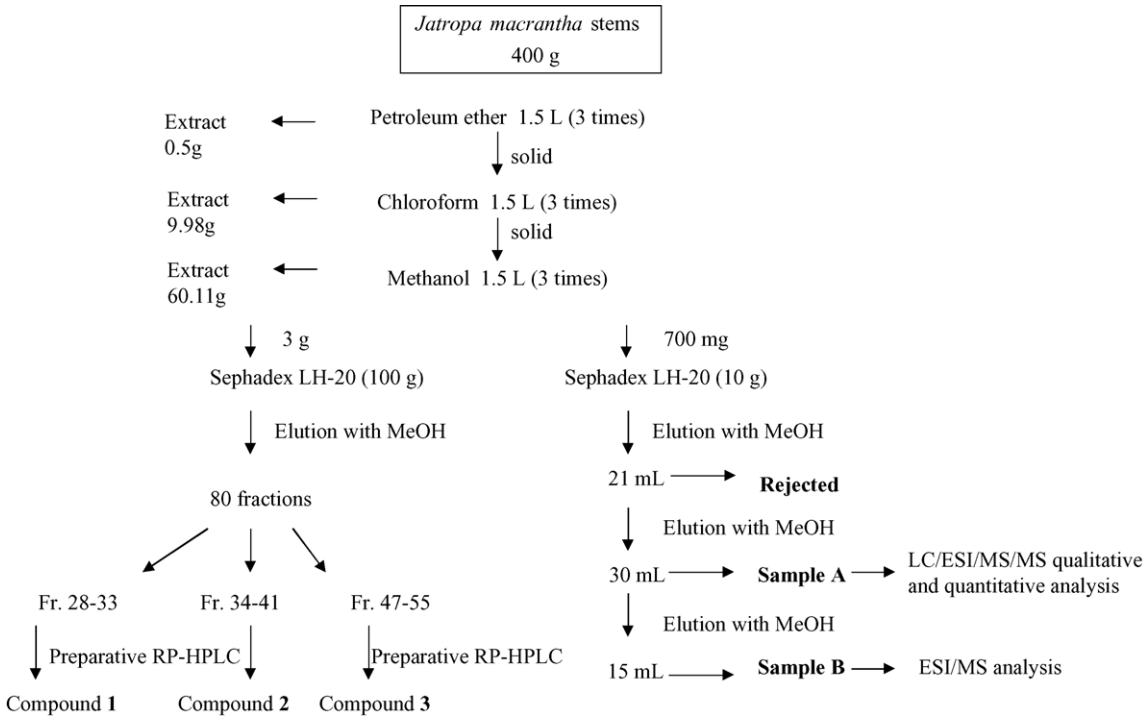


Fig. 1. Chemical structures of compounds **1**–**3** and internal standard (I.S.).



Scheme 1. Extraction, isolation and sample preparation procedures.

Fraction 47–55 (56.3 mg) were chromatographed by HPLC/DAD on a 300 mm × 10 mm i.d. Atlantis RP-D-C_{18} column (Waters) using H_2O/0.01% TFA as eluent A and CH_3CN/0.01% TFA as eluent B at a flow rate of 2.5 mL min^{-1} to afford compound **3** (11 mg, t_R = 25.08 min) (proanthocyanidin B-3, Fig. 1). An increasing linear gradient of solvent B was used, starting at 0% of B up to 100% in 50 min. Detection was carried out at 210 nm.

Isolation procedure is described in Scheme 1.

2.5. NMR analysis

NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) at 300 K. The NMR data were processed using UXNMR software.

^{1}H NMR data of compounds **1**–**3** in CD_3OD.

Catechin. ^{1}H NMR (CD_3OD, 600 MHz), δ: 2.52 (dd, H-4_{ax}, $J_{3,4ax}$ = 8.1 Hz, $J_{4ax,4eq}$ = 16.1 Hz), 2.86 (dd, H-4_{eq}, $J_{3,4eq}$ = 5.3 Hz, $J_{4ax,4eq}$ = 16.1 Hz), 3.99 (m, H-3), 4.57 (d, H-2, $J_{2,3}$ = 7.5 Hz), 5.87 (bs, H-6), 5.94 (bs, H-8), 6.73 (dd, H-6′, $J_{5',6'}$ = 8.0 Hz, $J_{2',6'}$ = 2.0 Hz), 6.77 (d, H-5′, $J_{5',6'}$ = 8.0 Hz), 6.85 (d, H-2′, $J_{2',6'}$ = 2.0 Hz).

*Catechin-7-O-β-*D*-glucopyranoside*. ^{1}H NMR (CD_3OD, 600 MHz), δ: 2.56 (dd, H-4_{ax}, $J_{3,4ax}$ = 8.1 Hz, $J_{4ax,4eq}$ = 16.2 Hz), 2.88 (dd, H-4_{eq}, $J_{3,4eq}$ = 5.3 Hz, $J_{4ax,4eq}$ = 16.2 Hz), 4.01 (m, H-3), 4.61 (d, H-2, J = 7.5 Hz), 6.17 (d, H-6, J = 2.2 Hz), 6.17 (d, H-8, J = 2.2 Hz), 6.73 (dd, H-6′, $J_{5',6'}$ = 8.1 Hz, $J_{2',6'}$ = 2.0 Hz), 6.78 (d, H-5′, $J_{5',6'}$ = 8.1 Hz), 6.85 (d, H-2′, $J_{2',6'}$ = 2.0 Hz), glucose unit: 4.83 (d, $H_{1'\text{-Glc}}$, $J_{1',2'}$ = 7.7 Hz), 3.42 (dd, $H_{2'\text{-Glc}}$, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 9.0 Hz), 3.46 (dd, $H_{3'\text{-Glc}}$, $J_{2',3'}$ = 9.0 Hz, $J_{3'-4'}$ = 9.0 Hz), 3.40 (dd, $H_{4'\text{-Glc}}$, $J_{3'-4'}$ = 9.0 Hz, $J_{4'-5'}$ = 9.0 Hz), 3.39 (m, $H_{5'\text{-Glc}}$), 3.71 (dd, $H_{6'\text{-Glc}}$, $J_{5'-6'}$ = 4.8 Hz, $J_{6'-6''}$ = 11.8 Hz), 3.90 (dd, $H_{6''\text{-Glc}}$ $J_{5'-6''}$ = 1.5 Hz, $J_{6'-6''}$ = 11.8 Hz).

Proanthocyanidin B-3. ^{1}H NMR (CD_3OD, 600 MHz), δ: 2.51 [dd, H-4_{ax} (F), $J_{3,4ax}$ = 8.1 Hz, $J_{4ax,4eq}$ = 16.1 Hz], 2.79 [dd, H-4_{eq} (F), $J_{3,4eq}$ = 5.7 Hz, $J_{4ax,4eq}$ = 16.1 Hz], 3.82 [m, H-3 (F)], 4.57 [d, H-2 (F), $J_{2,3}$ = 7.4 Hz], 6.10 [s, H-6 (D)], 6.28 [dd, H-6′ (E), $J_{5',6'}$ = 8.3 Hz, $J_{2',6'}$ = 2.0 Hz], 6.62 [d, H-2′ (E), $J_{2',6'}$ = 2.0 Hz], 6.70 [d, H-5′ (E), $J_{5',6'}$ = 8.3 Hz], 4.28 [d, H-2 (C), $J_{2,3}$ = 9.6 Hz], 4.38 [dd, H-3 (C), $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 7.9 Hz], 4.44 [d, H-4 (C), $J_{3,4}$ = 7.9 Hz], 5.82 [bs, H-8 (A)], 5.92 [bs, H-6 (A)], 6.50 [dd, H-6′ (B), $J_{5',6'}$ = 8.1 Hz, $J_{2',6'}$ = 2.0 Hz], 6.70 [d, H-5′ (B), $J_{5',6'}$ = 8.1 Hz], 6.77 [d, H-2′ (B), $J_{2',6'}$ = 2.0 Hz].

2.6. Preparation of phenolic standards and establishment of calibration curves

Stock solutions of each phenolic standard (l mg mL^{-1}) were prepared by dissolving each compound in MeOH. Five different solutions, containing respectively 5, 10, 25, 50 and 100 μg mL^{-1} of each compound (external standards) and 50 μg mL^{-1} of epigallocatechin, 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 between external standard and internal standard area against the know concentration of external standard. The result represents the average of three curves performed by three injection of each concentration.

2.7. Sample preparation

Sample preparation for analytical purpose was performed on Sephadex LH-20 in reduced scale. A sample of 700 mg of MeOH extract was fractionated on Sephadex LH-20 column (10 g), using MeOH as mobile phase. First elution with 21 mL of methanol afforded the fraction made up of low molecular weight compounds and salts; subsequent elution with 30 mL of methanol gave a fraction containing catechin, catechin-7-*O*-β-D-glucopyranoside, proanthocyanidin B-3 and trimeric proanthocyanidins (Sample A). The subsequent washing with 15 mL of methanol gave polymeric proanthocyanidins (Sample B). Compounds **1**–**3** were contained only in Sample A.

For qualitative purpose Sample A was diluted 1:10 with MeOH, and a volume of 10 μL was injected in the chromatographic system.

For quantitative purpose Sample A was diluted 1:10 with MeOH, added with internal standard (50 $\mu g\,mL^{-1}$) and a volume of 10 μL was injected in the chromatographic system.

For the analysis of polymeric proanthocyanidins, Sample B was diluted 1:100 and injected directly in the ESI source by using a syringe pump at the flow rate of 5 $\mu L\,min^{-1}$.

Sample preparation for analysis is summarised in Scheme 1.

2.8. ESI/MS and ESI/MS/MS analysis

ESI/MS and ESI/MS/MS analyses of the three standards, **1**–**3**, were performed using a Finnigan (Thermo Finnigan, San José, CA, USA) LCQ Deca ion trap instrument equipped with Xcalibur software. Samples of isolated compounds were dissolved in MeOH to obtain 1 $\mu g\,mL^{-1}$ solutions and infused into the ESI ionization source using a syringe pump at a flow rate of 5 $\mu L\,min^{-1}$. For the analysis of compounds **1**–**3**, the instrument was operating in the positive ion mode with a capillary voltage of 5 V, a spray voltage of 5 kV, and a tube lens offset of 20 V. Capillary temperature was 220 °C, sheath gas (N_2) flow rate was 60 (arbitrary units) and the data were acquired in the MS1 and MS/MS scanning modes. Scan range was *m*/*z* 120–900 in MS1 mode and 50–500 in MS/MS mode; maximum injection time was 50 ms, and number of microscan was 3, for MS/MS scanning mode percentage of collision energy was 30%, the activation time 30 ms. To tune the LCQ for catechin derivatives, the voltages on the lenses were optimised in the TunePlus function of the Xcalibur software in positive ion mode, whilst infusing a standard solution (l μg catechin/l mL MeOH) at the flow rate of 3 $\mu L\,min^{-1}$.

Full ESI/MS and CID (collision induced dissociation) ESI/MS/MS analyses of standard compounds, **1**–**3**, were performed for standards on API2000 Electrospray mass spectrometer equipped with a triple quadrupole analyzer (Applied Biosystems, Foster City, CA, USA). Optimised parameters consisted in declustering potential of 80 eV, focusing potential of 200 eV, entrance potential 9 eV. Experiments were run in Ql MS mode in order to obtain ESI-MS spectra and in product ion scan in order to obtain MS/MS experiments. When product ion scan was run, the collision energy was 50 eV, collision cell exit potential 3 eV. For optimising parameters a solution of catechin standard was infused in the source at the flow rate of 5 $\mu L\,min^{-1}$.

ESI/MS and ESI/MS/MS analyses of the high molecular weight proanthocyanidins (Sample B) were performed using a Finnigan (Thermo Finnigan, San José, CA, USA) LCQ Deca ion trap instrument equipped with Xcalibur software. Extract obtained by the purification step was infused into the ESI ionisation source using a syringe pump at a flow rate of 10 $\mu L\,min^{-1}$. For the analysis of polymeric catechins, the instrument was operating in the negative ion mode with a capillary voltage of −5 V, a spray voltage of 5 kV, and a tube lens offset of −20 V. Capillary temperature was 220 °C, sheath gas (N_2) flow rate was 60 (arbitrary units) and the data were acquired in the MS1 and MS/MS scanning modes. Scan range was *m*/*z* 100–2000 in MS1 mode when normal mass scan was performed, and scan range was 1000–4000 when high mass scan was performed.

2.9. LC–MS and LC–MS/MS

For qualitative purpose Sample A 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 symmetryshield (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 20% of solvent B in 15 min. After other 15 min the percentage of B became 35%. The flow generated by chromatographic separation was directly injected into the electrospray ion source. MS spectra were acquired and elaborated using the software provided by the manufacturer.

LC/ESI/MS/MS experiments were carried out by using dependent scanning mode, where the mass spectrometer software made a choice in real time about which ion to fragment and optimised all the parameters to do this based on the charge and on the mass.

For quantitative purpose, standards and Sample A were analysed by a 1100 HPLC system (Agilent, Palo Alto, CA) coupled with a triple quadrupole instrument API2000 (Applied Biosystems, Foster City, CA, USA). Chromatographic conditions were the same as described above for the LC/ESI/MS experiment. The instrument was used in the tandem MS mode, multiple reaction monitoring (MRM).

API2000 ESI source was tuned with a catechin standard solution in methanol (l μg/l mL) infused at the flow rate of 10 $\mu L\,min^{-1}$ with a syringe pump. Optimised parameters consisted in declustering potential of 80 eV, focusing potential of 200 eV, entrance potential 10 eV, collision energy 50 eV, collision cell exit potential 3 eV. The selected fragmentation reaction was the Retro Diels Alder (RDA) reaction characteristic for catechins, for all the three compounds.

Chromatographic conditions were the same described above.

3. Results and discussion

3.1. Qualitative LC–ESI-MS and LC–ESI-MS/MS

Phytochemical investigation on methanol extract of the stems of *J. macrantha* led to the isolation of catechin, catechin-7-*O*-β-D-glucopyranoside and proanthocyanidin B-3, reported in Fig. 1.

In order to realise a qualitative analysis on the catechin derivatives in *J. macrantha* extracts MS experiments were performed on Sample A by using an LC–MS system equipped with an ESI source and an Ion Trap analyser.

Positive ion electrospray LC/MS analysis, total ion current (TIC) profile and reconstructed ion chromatograms (RICs), of Sample A from *J. macrantha* stems are shown in Fig. 2. Catechin derivatives were identified comparing retention times and *m*/*z* values in the total ion current chromatogram to those of the selected standards. Reconstructed ion chromatograms were obtained for each value of *m*/*z* observed for standard compounds (*m*/*z* 291, *m*/*z* 453, *m*/*z* 579) in order to improve the separation and the identification of the single compounds. LC/MS analysis of Sample A revealed the presence of other compounds related to catechins. In particular peaks at *m*/*z* 867 were observed and the reconstructed ion chromatograms showed the presence of three peaks. The value of the mass of the pseudomolecular ion, the retention time and the presence in this Sephadex fraction suggested for these compounds the nature of trimeric proanthocyanidins.

In order to characterise these unexpected compounds and to reveal the presence of other correlated molecules in the extract, simultaneous experiments of LC/ESI/MS in selected ion monitoring (SIM) mode and LC/ESI/MS/MS, selecting the same ions as precursors, were performed.

The *m*/*z* values selected were *m*/*z* 579, in order to produce a fragmentation pattern for proanthocyanidin B-3 and *m*/*z* 867, in order to confirm the nature of trimeric proanthocyanidins and to analyse the different MS/MS spectra of the three compounds detected. Results of this experiment are reported in Fig. 3.

The chromatographic profile obtained from the LC/ESI/MS SIM experiment for the ion at *m*/*z* 579 showed the same profile of the reconstructed LC/MS chromatogram obtained for this ion in the previous experiment (Fig. 3A). In particular it showed one peak at retention time of 18.11 min that was attributed to compound **3**. The tandem mass spectrum produced by this compound (Fig. 3B) showed a major ion fragment at *m*/*z* 426.8, probably corresponding to a Retro Diels–Alder reaction, as described in literature for proanthocyanidins [20]. Loss of a neutral fragment

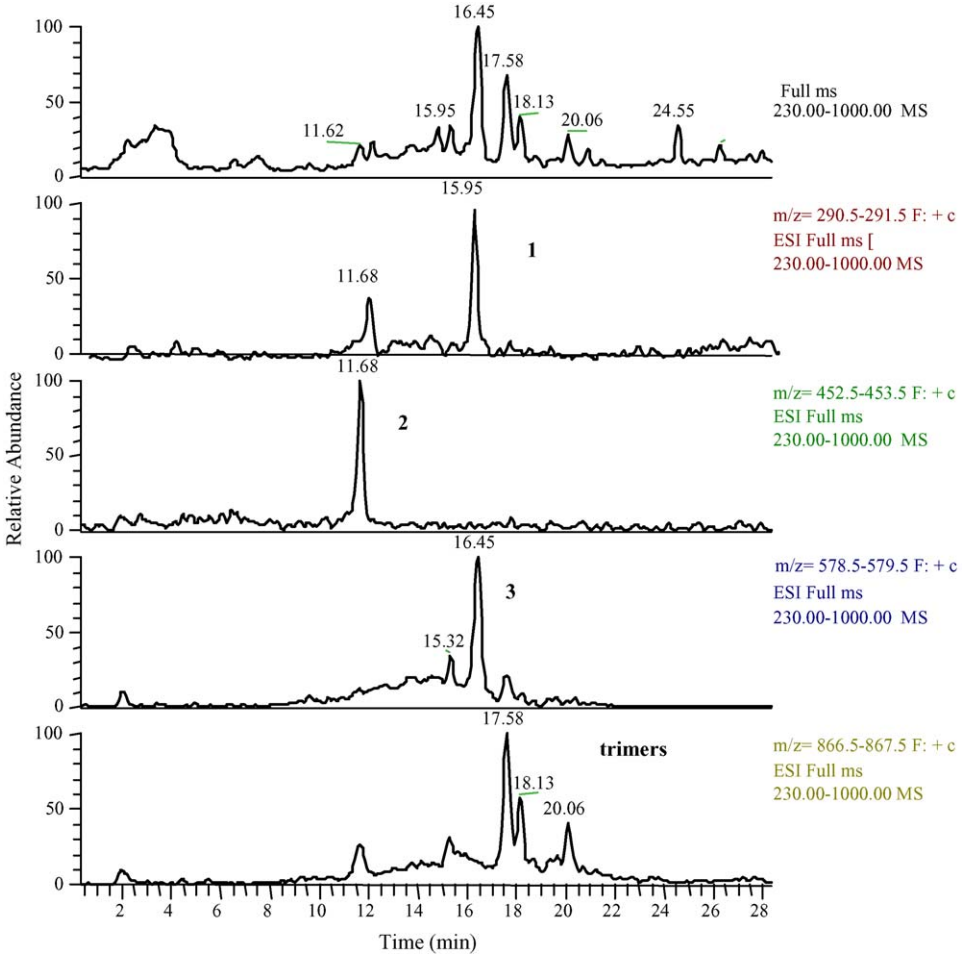


Fig. 2. LC–ESI-MS of Sample A from MeOH extract of *Jatropha macrantha* stems: catechin (**1**), catechin-7-*O*-β-D-glucopyranoside (**2**), proanthocyanidin B-3 (**3**). Column symmetry shield RP-C18. Gradient: time 0 min, 100% A; time 15 min, 20% B; time 35 min, 40% B.

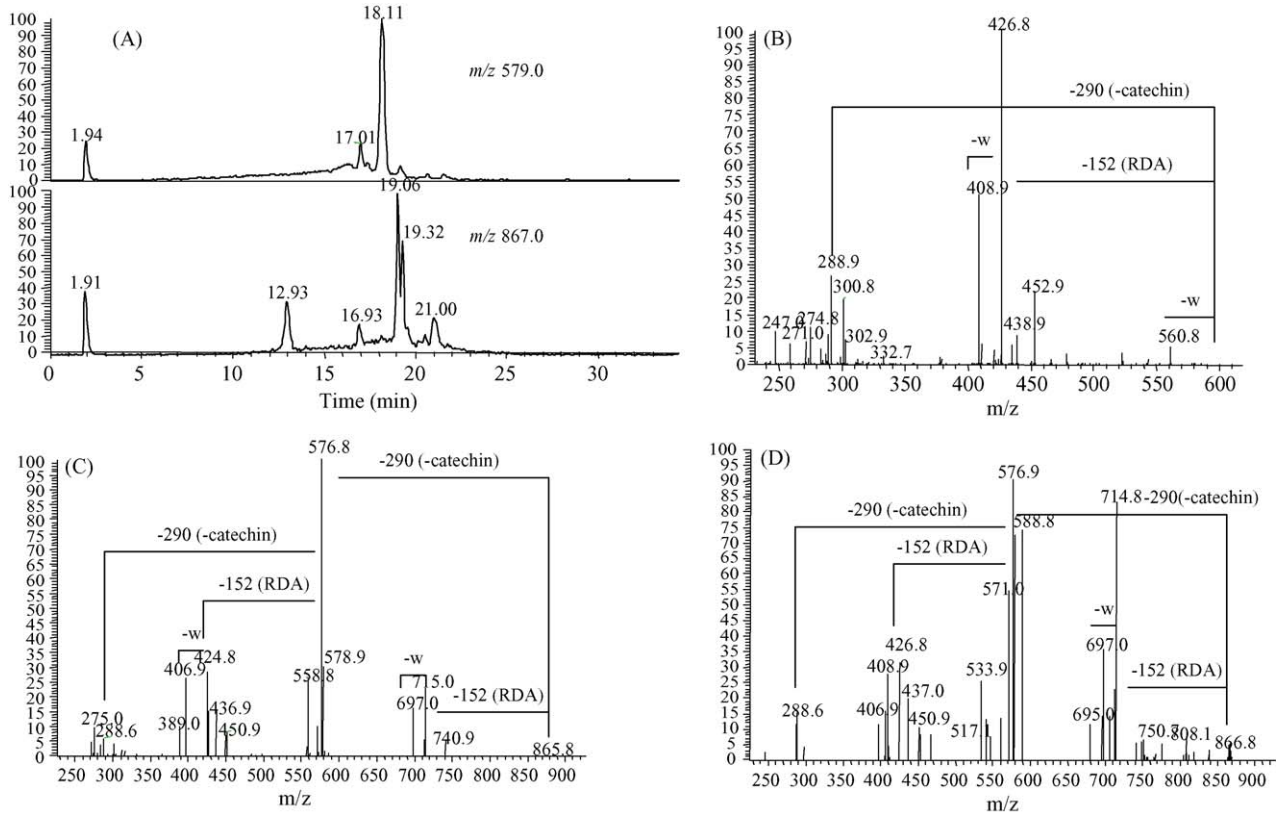


Fig. 3. LC–MS/MS product ion scan of dimeric and trimeric proanthocyanidins: (A) SIM profiles for *m*/*z* 579.0 and 867.0; (B) MS/MS spectrum of the ion at *m*/*z* 579.0 appearing at retention time 18.11 min; (C) MS/MS spectrum of the ion at *m*/*z* 867.0 appearing at retention time 19.06 min; (D) MS/MS spectrum of the ion at *m*/*z* 867.0 appearing at retention time 21.00 min.

at 152 amu (atomic mass unit) is a characteristic product of this reaction. Another fragment is present at the value of *m*/*z* 408.9, corresponding to the subsequent lost of a water molecule. Finally the ion at 288.9, corresponding to the breaking of the C–C linkage between the two catechin units, confirmed the nature of dimeric catechin of this compounds. The fragmentation pattern was used as a model to hypothesise the presence of trimeric proanthocyanidins in the extract, and specifically to hypothesise the nature of trimeric proanthocyanidin for compounds occurring as pseudomolecular ions at *m*/*z* 867.0. The chromatographic profile obtained from the LC/ESI/MS experiment for the ion at *m*/*z* 867.0 revealed the presence of three peaks at different retention times (Fig. 3A), and in addition an unidentified peak at retention time 12.93 min not related to catechins. Because of the close retention time between the compounds at retention time 19.06 and 19.32 min we did not obtain two different MS/MS spectra. Furthermore, the differences observed between the spectra obtained from the fragmentation of the compound occurring at retention time 19.06 min and the compound occurring at retention time 21.00 min was not enough marked to permit a structural characterisation. Analysis of the tandem mass spectra of the three different species, in the simultaneous LC/MS/MS experiment, showed the same fragment ions at different intensities. Both these compounds showed loss of one water molecule, RDA fragmentations and loss of one or two catechin units. This finding allowed us to confirm the nature of trimeric proanthocyanidins for these compounds.

3.2. ESI-MS of oligomeric proanthocyanidins in J. macrantha extract

The presence of trimeric proanthocyanidins in the extract suggested us the presence of polymeric proanthocyanidins. With the aim of a qualitative characterisation of these compounds in *J. macrantha* methanolic extract, Sample B obtained by the column chromatography separation step, was injected directly in the ESI-MS source. For the analysis of these compounds negative ionisation was preferred, expecting for each compound a pseudomolecular ion $[M-H]^-$ and unimportant fragmentation, on the basis of several reports from literature [21–23]. Fig. 4 shows the ESI-MS spectra for the condensed tannins (proanthocyanidins) extracted from the stems. Panel A reports the spectrum collected at low mass values (range 200–2000) and panel B reports the spectrum collected at high mass values (range 1000–4000).

ESI/MS analyses showed the presence of oligomers for each polymerisation level until 11 catechin units. Two factors prevent us to see more complex oligomers, one is the technical mass range of the mass analyser used, and the other is the evidence that, when the degree of polymerisation of the compounds increase, the pseudomolecular ion peaks become smaller and approach the background noise, which makes it difficult their identification.

ESI-MS mass spectrometry allowed to obtain fast and very informative results about condensed tannins using a very small

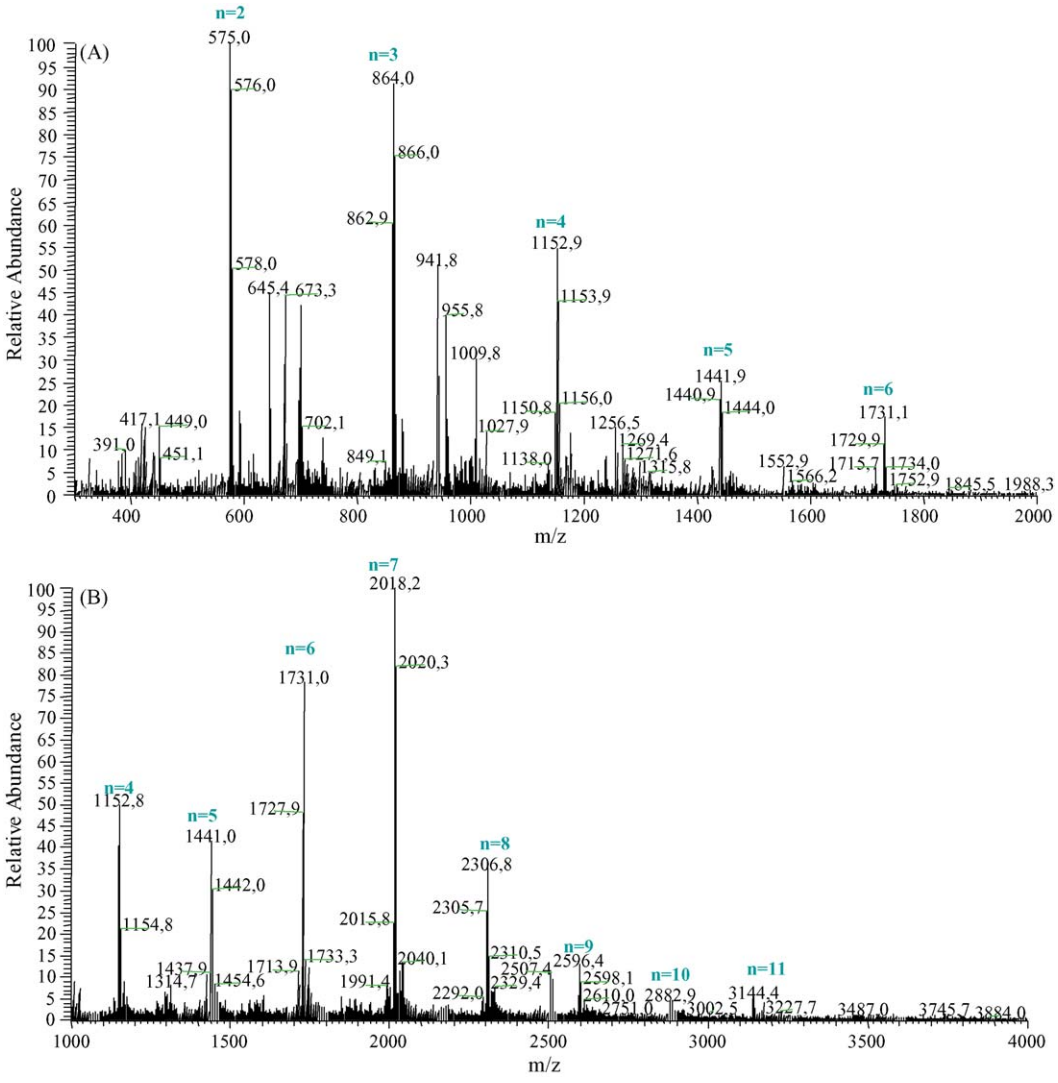


Fig. 4. ESI-MS of Sample B, polimeric proanthocyanidins, from *J. macrantha*: (A) normal mass scan; (B) high mass scan.

amount of extract. On the basis of these spectrometric data in *J. macrantha* stems extract proanthocyanidins exist with an extremely wide molecular weight range, from 290, corresponding to catechin, to 3144, corresponding to the oligomer generated from the condensation of 11 catechin units, and all the MW intermediates are present. This finding is very interesting considering that extracts rich in condensed tannins have been recently reported in literature to exert sexual stimulant activity and therapeutical activity in infertility; this is in agreement with the traditional use as an aphrodisiac of the plant under investigation.

3.3. Quantitative analysis of catechin derivatives in J. macrantha stems

In order to obtain an accurate quantitative determination of compounds **1**–**3** a quantitative LC–MS/MS method was developed. Since the fragment at *m*/*z* 139.0 is reported in literature [24] as specific for all the catechins and catechin derivatives, ESI-MS/MS analyses were recorded for the three standard compounds by using an LC–MS equipped with an ESI source and a triple quadrupole analyser. MS/MS spectra of catechin (A), catechin-7-*O*-β-glucopyranoside (B) and proanthocyanidin B-3 (C) obtained with triple quadrupole analyser are reported in Fig. 5. All the spectra presented the characteristic fragment originated by the reaction reported in Fig. 5D. Thus an MRM method was developed. Transition from the specific molecular weight of each compound to the fragment at *m*/*z* 139.0 was selected as specific reaction to monitor for all the catechin derivatives and for the internal standard, epigallocatechin.

Fig. 6 shows the MRM analyses of Sample A, spiked with internal standard at the concentration reported in the experimental. The chromatographic profile contained all the peaks corresponding to the compounds under investigation, with appreciable intensity for quantitative purpose.

3.3.1. Validation

Validation of the method was realised in agreement with EMEA note guidance on validation of analytical methods [25].

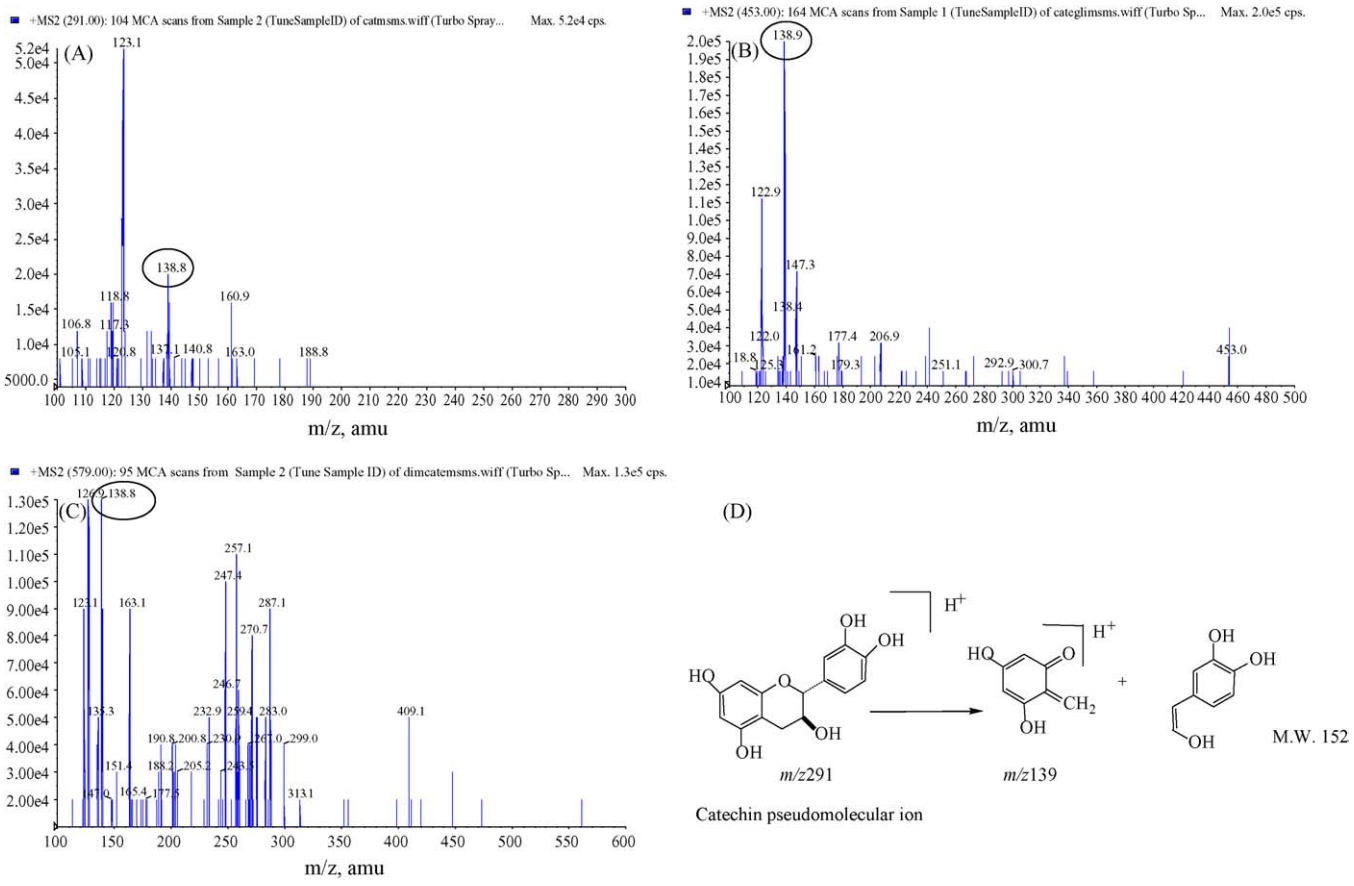


Fig. 5. ESI-MS/MS spectra of compounds **1** (A), **2** (B) and **3** (C) and Retro Diels–Alder reaction described for catechin (D).

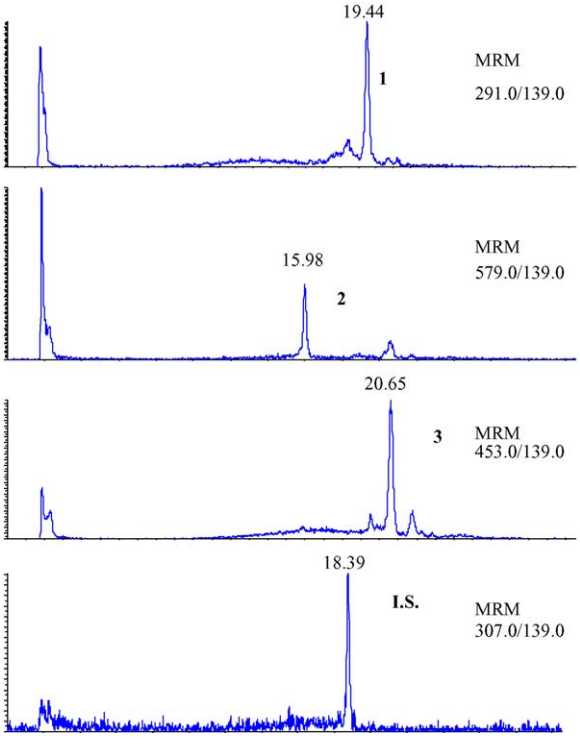


Fig. 6. LC–MS/MS of compounds **1**–**3** and internal standard (I.S.: epigallocatechin) in MRM mode.

Validation of the LC/MS/MS method included intra and inter-day precision and accuracy studies on 3 days. Accuracy and precisions were calculated by analysing three samples of each compound at three different concentration levels: 5, 25 and 100 $\mu g\,mL^{-1}$. Standard deviations calculated in this assay were <3% for all the three compounds under investigation.

The specificity is the non interference with other substances detected in the region of interest; the LC–MS/MS method, developed by using a characteristic fragmentation of catechins and derivatives, resulted to be specific with no any other peak interfering at the retention times of the three marker compounds in the MS/MS detection mode.

Recoveries were determined by the addition of known quantities of the three compounds under investigation to known amount of *J. macrantha* samples. Quantities were calculated by subtracting total amount of each compound before spiking to the total amount after spiking. Ratio between detected amount and spiked amount was used to calculate the recovery.

The mean recovery of the method was $100 \pm 2\%$.

The calibration graphs, obtained by plotting area ratio between external and internal standard versus the known concentration of each compound, were linear in the range of 5–100 $\mu g\,mL^{-1}$ for all compounds. Correlation values are reported in Table 1.

Quantification limit was measured to establish the sensitivity of the method. Quantification limit is defined as the lowest con-

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

Compound	r^2	t_R (S.D.)	LOQ (S.D.) μg mL^{-1}	Stems concentration (S.D.) mg kg^{-1}
1	0.9981	19.45 (0.05)	0.65 (0.02)	441.52 (3.13)
2	0.9980	15.98 (0.05)	0.98 (0.05)	220.21 (2.88)
3	0.9986	20.68 (0.06)	1.02(0.11)	1259.11 (8.71)

r^2: regression value for the calibration curve; t_R: retention time as a mean of the different values recorded, $n = 5$; LOQ: limit of quantification; stems concentration: mg kg^{-1} of dried stems, $n = 5$.

centration 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.

LOQ values for the three compounds are reported in Table 1.

3.3.2. Analyses of J. macrantha samples

Five aliquots of Sample A were analysed in order to quantify the catechin derivatives content. Table 1 report quantisation data for compounds **1**–**3**, regression of calibration curves, variability in retention times and quantitative values in dried stems of *J. macrantha*.

Quantitative analyses results confirmed that compounds **1**–**3** are major compounds of the plant and, in particular, proanthocyanidin B-3 appears to be the most abundant. Unfortunately on the basis of the absence of commercial samples for trimeric proanthocyanidins as standards, quantitative analysis was not performed for these compounds.

4. Conclusions

The presence of catechin derivatives was never reported for this plant and it is an interesting finding considering the possible role of catechin and its polymeric derivatives in the biological activity connected with the traditional use of the stems.

The ESI-MS method allowed us to define the presence of catechin oligomers and to characterise a number of compounds of different polymerisation degree with a single fast analysis. These compounds are reported to possess a lot of different attractive biological activities.

Commercial preparation based on *J. macrantha* recently introduced in the International herbal market will contribute to the need of methods for industrial quality control in order to quantify marker compounds in raw materials and in final products. The quantitative method described in this paper is also straightforward and convenient because it requires a very fast sample preparation procedure.

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