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Strong antioxidant phenolics from *Acacia nilotica*: Profiling by ESI-MS and qualitative–quantitative determination by LC–ESI-MS

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ABSTRACT

Acacia nilotica (L.) Del. syn is a species rich in polyphenolic constituents, in which catechins are hypothesized to possess antioxidant properties and to play a role in the anti-inflammatory activity of several plants. Due to the complexity of catechin derivatives, the investigation of this class of natural compounds has been limited by difficulties in their separation. In this paper, rationalization of the phenolics occurring in the 80% EtOH extract of *Acacia nilotica* pods, on the basis of ESI-MS and ESI-MS/MS profiles, has been proposed. Additionally, an LC–ESI-MS qualitative study has been performed by using a C18 polar endcapped stationary phase. The fragmentation pattern obtained evidenced the presence in *A. nilotica* pods of galloylated catechin- and gallocatechin derivatives along with galloylated glucose derivatives. The structures were confirmed by NMR, after isolation of the pure compounds.

In addition, the radical scavenging activities of extracts and pure compounds were investigated, by using the TEAC assay. Furthermore quantitative analyses were performed by LC–ESI-MS/MS, confirming the interest of this species as a rich source of very strong antioxidant principles.

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1. Introduction

Acacia nilotica Lamarack (*Mimosa nilotica*) is a medium size tree locally named also “Babul” or “Kikar”, belonging to the Mimosaceae family. It is a native from Egypt to Mozambique and grows in tropical and subtropical countries, in particular is quite abundant in Pakistan [1], where the plant is used in folk medicine [2,3]. Traditionally the bark, leaves, pods and fruits are employed against diarrhea, fever, tuberculosis and menstrual problems [2,3]. The plant is also reported to exhibit anti-inflammatory [4], and antiplatelet [5] activities. Methanol (bark and pods) and aqueous (pods) extracts are reported to exert inhibitory effects against HIV-1 protease [6] and hepatitis C virus protease [7].

Phytochemical studies of the aerial parts of the plant resulted in the identification of a variety of phenolic constituents, among which catechin derivatives [3,5,6,8] were identified.

These compounds have a wide range of biological activities, in particular antioxidant, anticarcinogenic and anti-inflammatory activities [9,10]. Anti-inflammatory activity described for these molecules is probably related to their antioxidant properties [11] and to their inhibition of arachidonic acid metabolism via cyclooxygenase and lypooxygenase pathways [12].

In the last years, many studies were directed to pycnogenol® (PYC), a patented standardised extract from the bark of the French maritime pine, *Pinus pinaster* [13], showing as the main constituents, the monomeric phenolic compounds (catechin, epicatechin and taxifolin) and proanthocyanidins based on catechin and epicatechin subunits [14]. A variety of biological activities are reported for pycnogenol, in particular antioxidant and anti-inflammatory activities [15,16].

Due to the structural complexity of catechin derivatives and high difficulties in their separation, studies on these compounds are limited in comparison with other polyphenolics [17]. Usually, catechins give unresolved HPLC peaks, due to the similarity in their structures and also to the large number of phenolic groups, that can give the same interactions with chromatographic stationary phase [18].

The development and the availability of effective Liquid Chromatography–Mass Spectrometry (LC–MS) and the Multiple Mass Spectrometry (MS/MS and MS^n) systems have supplied very useful tools to determine catechin derivatives in complex natural matrixes [19,20].

In the present work, rationalization of the presence of the polyphenolic constituents in *A. nilotica* pods on the basis of ESI-MS

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and ESI-MS/MS profiles has been proposed. Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry has been used to investigate the presence of these compounds in the 80% EtOH extract of *A. nilotica* pods.

In the second stage, analytical HPLC–ESI-MS method has been developed. Since catechin derivatives are very complex to separate with classical C18 stationary phases, in our investigation a monolithic C18 column, a stationary phase described to produce better resolution in different analytical fields, was used [21]. Monoliths are rod-shaped continuous bed silica or polymeric materials, which offer an alternative to conventional particle-packed columns for analytical and preparative liquid chromatography.

Additionally, the antioxidant activity of fractions and isolated compounds has been evaluated by measuring their ability to scavenge the radical cation 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) in the TEAC assay. Furthermore, an LC–ESI-MS/MS method has been developed to quantify the single constituents in *A. nilotica* pods.

2. Materials and methods

2.1. Materials

HPLC grade methanol (MeOH), acetonitrile (ACN) and acetic acid (CH_3COOH) 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).

2.2. Chemicals

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, sodium chloride, monobasic sodium phosphate and dibasic sodium phosphate were obtained from Sigma–Aldrich (Gillingam, Dorset, UK). The solvents were obtained from Carlo Erba reagent (Milano, Italy). Nanopure water was prepared by Milli-Q apparatus.

2.3. Plant material

The pods of *Acacia nilotica* (L.) (Qarad) were collected in May 2008 in Aswan, south of Egypt and identified by Prof. Arafa Hamed according to Täckholm (1974). The voucher specimens are deposited in the Botany Department herbarium, Aswan Faculty of Science, Egypt.

2.4. Extraction and sample preparation

The pods of *Acacia nilotica* (L.) (Qarad) (250 g) were powdered and exhaustively extracted with 80% EtOH by maceration at room temperature. The crude extract was concentrated under reduced pressure to a syrupy consistence (50 g). 1.5 g from the crude extract was dissolved in a small quantity of H_2O and was loaded on a water preconditioned short C18 column (6 cm × 10 cm, packed with kLiChroprep RP-18, 40–60 lm, Merck) and eluted with H_2O (ANP-1, 127 mg), 20% MeOH (ANP-2, 393 mg), 60% MeOH (ANP-3, 115 mg), and 100% (ANP-4, 85 mg), respectively.

2.5. Equipment

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 a reversed phase HPLC C18 Atlantis column 250 mm × 10 mm (Waters, Milford, MA).

IT-ESI-MS, IT-ESI-MS/MS and LC–ESI-MS analyses were performed using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. Chromatography was performed on an RP C18 monolithic Onyx and RP C18 Atlantis T3 columns.

UV spectra were recorded on a UV-2101PC, UV–vis scanning spectrophotometer (Shimadzu Italia srl, Milan, Italy).

2.6. MALDI-MS, ESI-MS and ESI-MS/MS analyses

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 α-cyano-4-hydroxycinnamic acid at 190.0504 Da as internal standard.

Full scan ESI-MS and collision induced dissociation (CID) ESI-MS/MS analyses of samples were performed on a Thermo Electron (San Josè, CA, USA) LCQ Deca IT spectrometer equipped with an ion trap analyser. Samples were infused directly into the source at a flow rate of 5 μL/min. The capillary voltage was −4 V, the spray voltage was 5 kV, the capillary temperature was at 270 °C, sheath gas (nitrogen) flow rate was 80 (arb) and auxiliary gas flow rate was 5 (arb). Data were acquired in the negative ion MS and MS/MS modes in the range of 250–1000 *m/z*.

2.7. Isolation and identification of compounds from A. nilotica 80% ethanolic extract

ANP-2 (393 mg) fraction was loaded on a small C18 column (3 cm × 30 cm, LiChroprep_RP-18, 25–40 lm, Merck) and eluted with 20% MeOH to give fractions **I** (50 mg), **II** (15 mg), **III** (15 mg) and **IV** (195 mg). ANP-3 (115 mg) fraction was loaded on a C18 column (3 cm × 30 cm, packed with LiChroprep_RP-18, 25–40 lm, Merck) and eluted with 35% MeOH to give purified fraction **V** (25 mg).

Successively, fractions **I**–**V** were chromatographed by HPLC-UV. Separations were carried out using a C18 Atlantis column 10 mm × 200 mm, particle size 10 μm (Waters, Millford, MA) eluted with mixtures of water containing 0.1% trifluoroacetic acid (TFA; solvent A) and acetonitrile containing 0.1% TFA (solvent B) at a flow rate of 2 mL/min.

From fraction **I** compound **1** (3.1 mg, t_R = 20.7) was obtained. The elution gradient was: 0–5 min, from 5% B to 10% B; 5–15 min, isocratic elution; 15–30 min, from 10% B to 40% B. From fraction **II** compounds **2** (4.2 mg, t_R = 21.7) and **3** (9 mg, t_R = 24.2) were obtained. The elution gradient was: 0–10 min, from 10% B to 15% B; 10–25 min, isocratic elution; 25–50 min, from 15% B to 50% B. Fraction **III** yielded compound **4** (2.5 mg, t_R = 40.5). The elution gradient was: 0–10 min, from 10% B to 15% B; 10–25 min, isocratic elution; 25–50 min, from 15% B to 50% B. From sample **IV** compounds **5** (2.4 mg, t_R = 14.6), **6** (42.0 mg, t_R = 17.1), **7** (34.3 mg, t_R = 22) and **8** (5.1 mg, t_R = 20.3) were obtained. The gradient started from 40% of B, to achieve 60% of B in 50 min and reached 100% B in the subsequent 10 min. From sample **V** compounds **9** (5.2 mg, t_R = 24.7) and **10** (4.5 mg, t_R = 31.3) were obtained. The elution gradient was: 0–5 min, from 5% B to 10% B; 5–15 min, isocratic elution; 15–30 min, from 10% B to 40% B. The selected detection wave-lengths were 280 nm, 254 nm and 350 nm.

2.8. NMR analysis

Optical rotations were measured on a JASCO DIP 1000 polarimeter.

Table 1
^{13}C and ^{1}H NMR spectroscopic data of compound **4** in CD_3OD.

Compound **4**		
Position	^{13}C	^{1}H
2	82.8	4.67 (*d*; *J* = 7.0 Hz)
3	68.2	4.08 *m*
4	27.8	2.64 (*dd*; *J* = 6.2, 15.8 Hz) 2.91 (*dd*; *J* = 4.5, 15.8 Hz)
4a	106.7	
5	151.7	
6	101.6	6.24 *s*
7	157.3	
8	101.6	6.24 *s*
8a	156.4	
1′	131.0	
2′	106.8	6.44 *s*
3′	146.5	
4′	133.9	
5′	146.5	
6′	106.8	6.44 *s*
Galloyl unit		
1″	120.5	
2″	110.2	7.19 *s*
3″	146.5	
4″	140.2	
5″	146.5	
6″	110.2	7.19 *s*
7″	166.9	

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 (99.95%, Sigma–Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra. The ROESY spectra were acquired with t_{mix} = 400 ms. The NMR data were processed using UXNMR software.

All the compounds were isolated and characterized by NMR and MS data in comparison with those reported in literature [8,22–24].

Gallocatechin 7-O-gallate (**4**): yellow powder; $[\alpha]^{25}_{D}$ +56.74 (*c* 0.2416, MeOH); UV (MeOH) λ_{max} 280 nm, HRMALDI-MS *m/z* 457.0788 [M−H]⁻ (calcd. for $C_{22}H_{17}O_{11}$ 457.0771). For ^{1}H and ^{13}C NMR data in CD_3OD; see Table 1.

2.9. LC–ESI-IT-MS and LC–ESI-QqQ-MS/MS analyses

For qualitative purpose ANP-2 and ANP-3 were analysed by LC/ESI/MS "on-line" using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. For ANP-2 chromatography was performed on an Onyx Monolithic C18 column (Phenomenex, USA), 100 mm × 3.0 mm. A gradient elution was performed by using a mobile phase A represented by water acidified with CH_3COOH (0.1%) and a mobile phase B represented by acetonitrile acidified with CH_3COOH (0.1%). The gradient started from 0% of eluent B to achieve 30% of B in 55 min. For ANP-3, a gradient elution based on a mobile phase A represented by water acidified with TFA (0.1%) and a mobile phase B represented by acetonitrile acidified with TFA (0.1%) were used. Elution was by step gradient from 100% of eluent A to 20% B in 15 min, then from 20% B to 40% B in 20 min, and subsequently to 100% B in 15 min. Chromatography was performed on an RP C18 Atlantis T3 column 2.1 mm × 150 mm (Waters, Millford, MA).

The flow (250 μL/min) generated by chromatographic separation was directly injected into the electrospray ion source.

The mass spectrometer was operating in the negative ion mode under the following conditions: capillary voltage −7 V, spray voltage 5 kV, tube lens offset 10 V, and capillary temperature 280 °C and sheath gas (nitrogen) flow rate 80 (arb). MS spectra were acquired in the range of 250–1000 *m/z* and elaborated using the software provided by the manufacturer.

Quantitative on-line HPLC–ESI-MS/MS analysis of the extract were performed using an Agilent 1100 HPLC system interfaced to a Applied Biosystems (Foster City, CA, USA) API2000 instrument. A gradient elution was performed by using a mobile phase A represented by water acidified with formic acid (0.1%) and a mobile phase B represented by acetonitrile acidified with formic acid (0.1%). The gradient started from 0% of eluent B, then from 0% B to 25% B in 15 min, and subsequently to 70% B in 25 min. The flow (200 mL min^{-1}) generated by chromatographic separation was directly injected into the electrospray ion source. Chromatography column used was an RP C18 Atlantis T3 150 mm × 2.1 mm (Waters, Milford, MA).

The API 2000 ES source was tuned by infusing a standard solution of catechin-7-gallate (**8**) (1 μg/mL in methanol) into the source at a flow rate of 10 μL/min. The optimised parameters were: declustering potential −172 eV, focusing potential −140.9 eV, entrance potential −4.2 eV, collision energy −30 eV, and collision cell exit potential −15 eV. The spectrometer was used in the MS/MS mode with MRM of fragmentation reactions selected for each compound as described below.

2.10. Calibration and quantification of phenolics

In order to prepare the calibration plot, a sample (1.0 mg) of each compound was weighted accurately into a 1 mL volumetric flask, dissolved in methanol and the volume made up to the mark with methanol. The resulting stock solution was diluted with methanol in order to obtain reference solutions containing 1, 5, 10, 20, 50 and 100 μg/mL of external standard.

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 curves performed by three injection of each concentration. All quantitative data were elaborated with the aid of Analyst software (Applied Biosystems).

2.11. Sample preparation

For qualitative purpose a solution 1 mg/mL of 80% EtOH extract was prepared and a volume of 20 μL was injected in the chromatographic system.

For quantitative purpose a solution 1 mg/mL of 80% EtOH extract, and a volume of 10 μL was injected in the chromatographic system.

2.12. TEAC test

Isolated compounds were tested by using the trolox equivalent antioxidant capacity (TEAC) assay. The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) by spectrophotometric analysis [25]. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The $ABTS^{\bullet+}$ solution was then diluted with PBS (pH 7.4) to obtain an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Samples were diluted with methanol to produce solutions of 0.3, 0.5, 1 and 1.5 mM for 1,3-di-*O*-galloyl-β-D-glucose (**1**), 1,6-di-*O*-galloyl-β-D-glucose (**2**), gallic acid methyl ester (**3**), gallocatechin-7-*O*-gallate (**4**), gallic acid methyl ester-4-gallate (**5**), and solutions of 0.1, 0.3, 0.5 and 1 mM for gallocatechin-7,3′-di-*O*-gallate (**6**), gallocatechin-7,4′-di-*O*-gallate (**7**), catechin-7-*O*-gallate (**8**), catechin-7,3′-di-*O*-gallate (**9**) and catechin-7,4′-di-*O*-gallate (**10**). Extract samples were prepared by dissolving 2 mg of dried

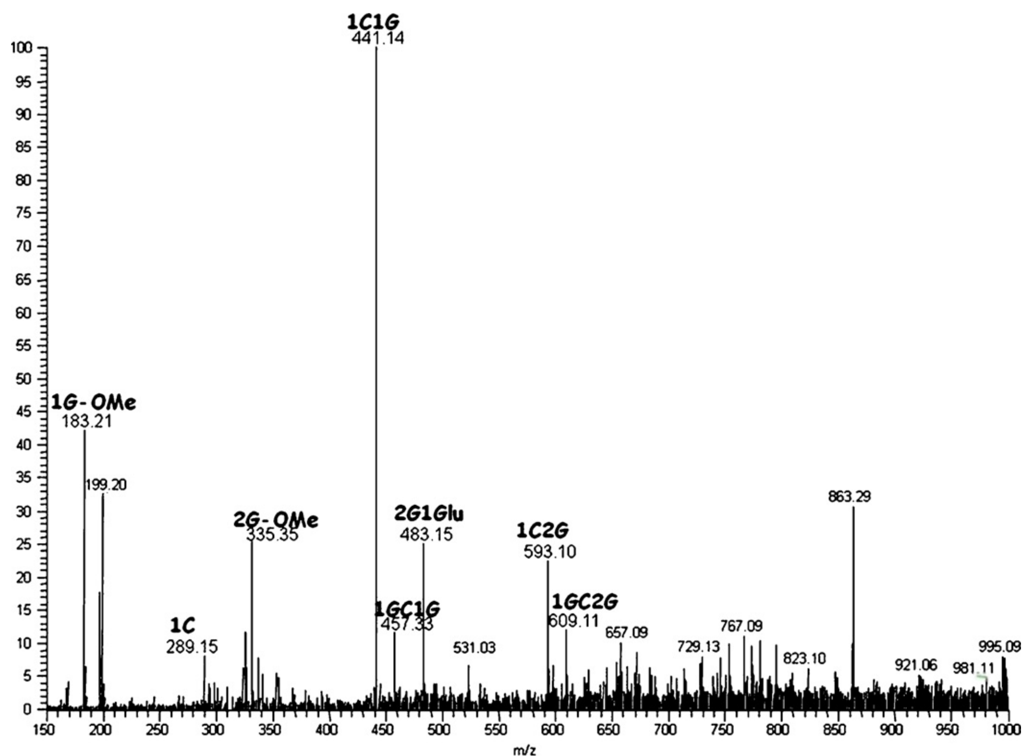

Fig. 1. ESI-IT-MS fingerprint (negative ion mode) of the 80% EtOH extract of *Acacia nilotica* pods.

extracts in 1 mL of methanol and then preparing 0.1, 0.2, 0,4 and 0.5 μg/mL operative solutions. The reaction was initiated by the addition of 1 ml of diluted ABTS to 10 μl of each sample solution. Determinations were repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of concentration of compound or standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

The antioxidant activities samples are expressed as TEAC values in comparison with TEAC activity of the reported reference compound quercetin [25]. The TEAC value is defined as the concentration of standard trolox with the same antioxidant capacity as a 1 mM concentration of the compound under investigation.

3. Results and discussion

3.1. ESI-MS and ESI-MS/MS fingerprint

With the aim of a rapid qualitative characterization of the phenolic compounds occurring in the 80% EtOH extract of *A. nilotica* pods, a preliminary ESI-MS analysis was performed. Fig. 1 shows the ESI-MS fingerprint in the negative ion mode. The obtained *m/z* values evidenced the presence of phenolics belonging to the classes of gallic acid, catechin and gallocatechin derivatives. Together with the ion relative to catechin at *m/z* 289, abundant ions corresponding to methylated gallic acid (G-OMe; *m/z* 183), methylated digallic acid (2G-OMe; *m/z* 335), catechin gallate (C1G; *m/z* 441), gallocatechin gallate (GC1G; *m/z* 457), digalloyl glucose (2GGlu; *m/z* 483), catechin digallate (C2G; *m/z* 593) and gallocatechin digallate (GC2G; *m/z* 609), respectively, were observed.

In order to obtain and analyze phenolic enriched fractions, a procedure of separation was developed as described above. ANP-1, ANP-2, ANP-3 and ANP-4 fractions were obtained and for further analyses, samples ANP-2 and ANP-3, that presented a major complexity and diversity, were selected (ANP-1 and ANP-4 were washing fractions).

Negative ionization was preferred since it has been demonstrated that it is more sensitive and selective for polyphenolic compounds [18]. Fig. 2 shows the deprotonated ESI-IT-MS fingerprint of ANP-2 fraction. The mass spectrum obtained evidenced the presence of phenolics belonging to catechin and gallocatechin series along with their galloyl derivatives. Together with the ions relative to catechin (C) at *m/z* 289 and gallocatechin (G1C) at *m/z* 305, ion peaks relative to monogalloyl catechin (C1G; *m/z* 441), gallocatechin gallate (GC1G; *m/z* 457) and gallocatechin digallate (GC2G; *m/z* 609), respectively, were observed. Mass spectra also showed ions corresponding to digalloyl glucose (2GGlu; *m/z* 483), trigalloyl glucose (3GGlu; *m/z* 635) and tetragalloyl glucose (4GGlu; *m/z* 787).

Fig. 3 shows the ESI-IT-MS fingerprint obtained for ANP-3 sample. The fragmentation pattern evidenced the presence of catechin together with its galloyl derivatives. Together with the ions relative to catechin (C) at *m/z* 289 and catechin gallate (CG) at *m/z* 441, ion peaks relative to catechin digallate (C2G; *m/z* 593), catechin trigallate (C3G; *m/z* 745) and catechin tetragallate (C4G; *m/z* 897), respectively, were observed.

In order to verify the hypothesis described above, second-order ESI-IT-MS/MS experiments for identified compounds were performed. Fig. 4 shows MS/MS of the precursor ions at *m/z* 335, *m/z* 441, *m/z* 457, *m/z* 483, *m/z* 635, *m/z* 745, *m/z* 787 and *m/z* 897 values.

The MS^2 spectrum of the ion at *m/z* 335 (Fig. 4A) showed only a major product ion $[M-152-H]^-$ at *m/z* 183, due to the loss of a gallic acid unit. The MS^2 spectrum of the ion at *m/z* 441 (Fig. 4B) showed a major product ion $[M-152-H]^-$ at *m/z* 289 attributable to the loss of a gallic acid unit. The MS^2 spectrum of the ion at *m/z* 457 (Fig. 4C) showed major product ions at *m/z* 439, 305 and 289; the first ion $[M-H-18]^-$ was due to the neutral loss of water; the second ion $[M-152-H]^-$ was relative to the neutral loss of a gallic acid unit, giving rise to a molecule of gallocatechin, confirmed by the presence of the third ion $[M-152-16-H]^-$ at *m/z*

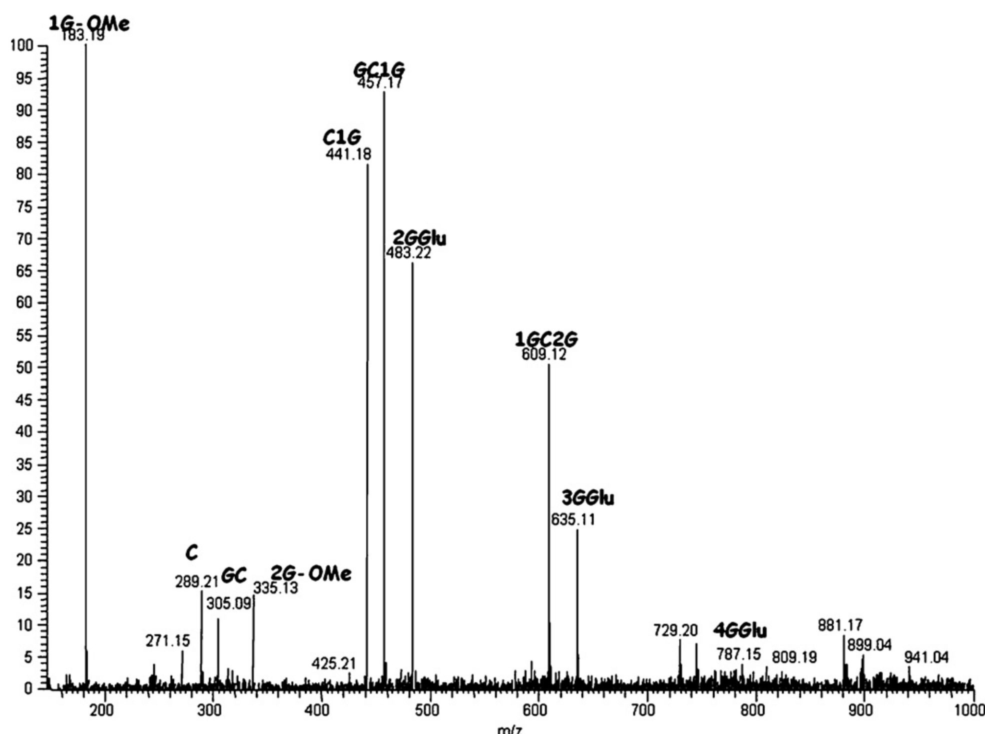

Fig. 2. ESI-IT-MS (negative ion mode) fingerprint of ANP-2 fraction.

289 attributable to a catechin unit. The MS^2 spectrum of the ion at *m/z* 483 (Fig. 4D) showed major product ions at *m/z* 331, 313 and 169 due to the loss of a gallic acid unit $[M-152-H]^-$, and to the subsequent losses of water $[M-152-18-H]^-$ and a glucose unit $[M-152-162-H]^-$. The MS^2 spectrum of the ion at *m/z* 635 (Fig. 4E) showed major product ions at *m/z* 483, 465 and 313, derived from the loss of a galloyl moiety $[M-152-H]^-$ followed by the neutral loss of water $[M-152-18-H]^-$ and of a second gallic acid unit $[M-152-18-152-H]^-$. The MS^2 spectrum of the ion at *m/z* 745 (Fig. 4F) showed major product ions at *m/z* 593 $[M-152-H]^-$ and 441 $[M-152-152-H]^-$, corresponding to the consecutive losses of two gallic acid units. The MS^2 spectrum of the ion at *m/z* 787 (Fig. 4G) showed a major product ion at *m/z* 635 due to the loss of a galloyl moiety $[M-152-H]^-$. The MS^2 spectrum of the ion at *m/z*

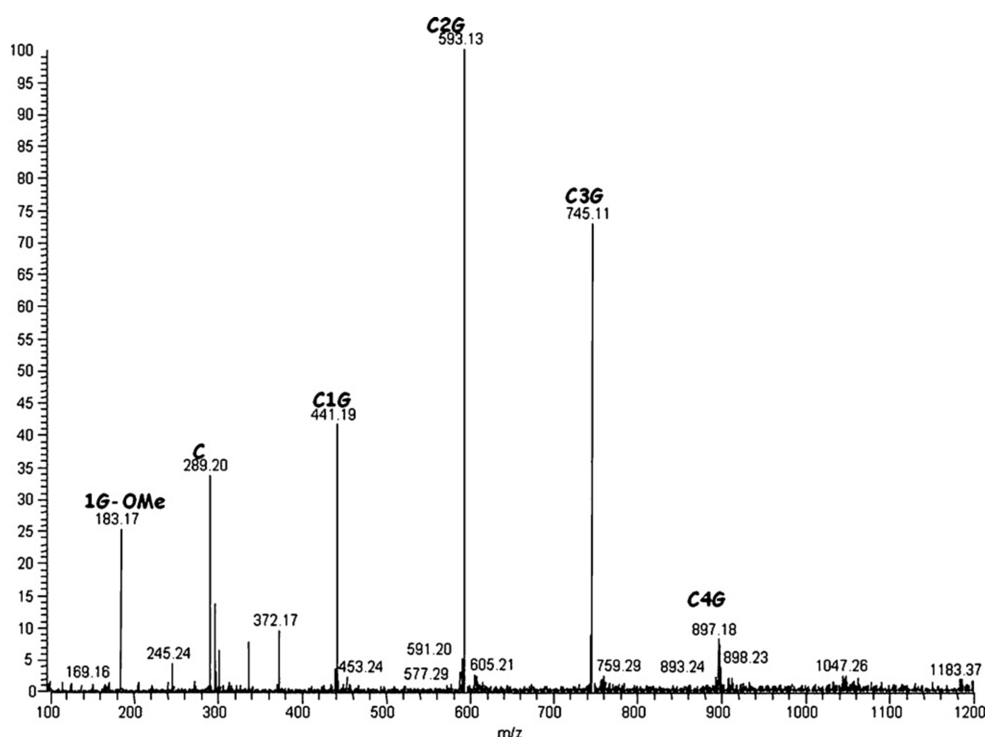

Fig. 3. ESI-IT-MS (negative ion mode) fingerprint of ANP-3 fraction.

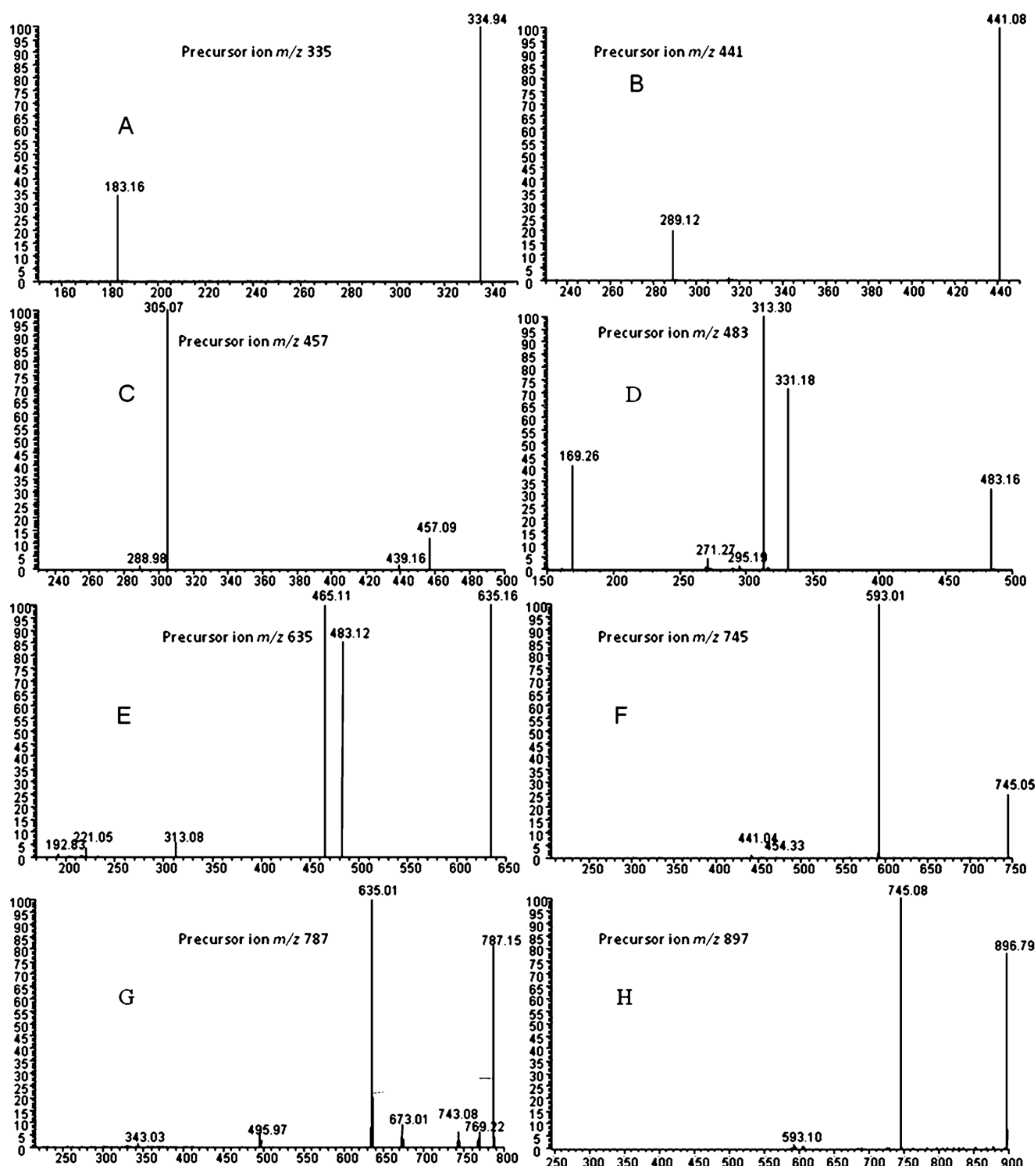

Fig. 4. ESI-IT-MS/MS (negative ion mode) spectra of *m/z* 335, 441, 457, 483, 635, 745, 787 and 897 values.

897 (Fig. 4H) showed major product ions at *m/z* 745 $[M-152-H]^-$ and 593 $[M-152-152-H]^-$, due to the consecutive losses of two gallic acid units.

3.2. LC–ESI-MS analysis

In order to investigate the presence of different compounds with the same molecular weight and then to realise a qualitative analysis on the polyphenolic constituents occurring in the 80% EtOH extract of *A. nilotica* pods, MS experiments were performed by using an LC–MS system equipped with an ESI source and an Ion Trap analyser. A full MS scan, in the form of a total ion current chromatogram (TIC), was acquired, and reconstructed ion chromatograms (RICs) were derived for each of the expected *m/z* values based on the molecular weights of the possible constituents.

Negative ion electrospray LC–ESI-MS analysis obtained in the acquisition range of 240–1400 amu (atomic mass unity), of ANP-2 and ANP-3 are shown in Figs. 5 and 6, respectively. Most of the compounds were efficiently separated in both samples, and it was possible to recognise peaks corresponding to the deprotonated molecular ions of identified compounds. RICs were generated for specific compounds expected to be present in the sample. For

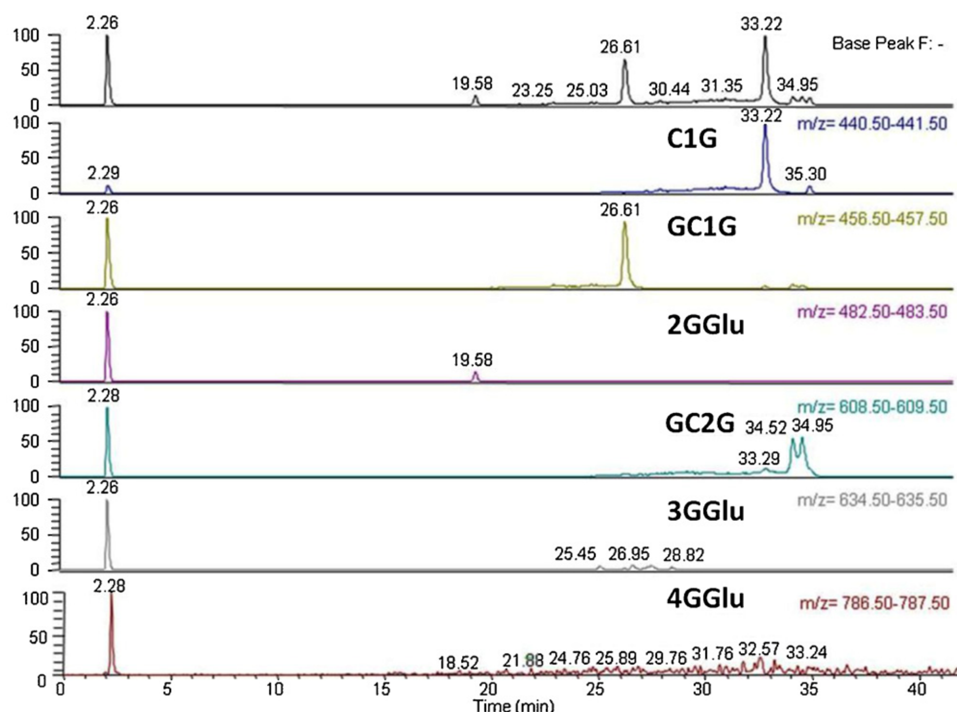

Fig. 5. LC–ESI-MS TIC (negative ion mode) (base peak in the range 250–1000 *m/z*) and RICs (reconstructed ion chromatograms) of ANP-2 fraction.

ANP-2, RIC relative to *m/z* 441 showed one peak at t_R = 33.20 min corresponding to catechin gallate, RIC relative to *m/z* 457 showed one peak at t_R = 26.61 min corresponding to gallocatechin gallate, RIC relative to *m/z* 483 showed a peak at t_R = 19.58 min, corresponding to digalloyl glucose, RIC relative to *m/z* 609 showed two peaks at t_R = 34.52 and 34.95 min, corresponding to two different gallocatechin digallate derivatives, RIC relative to *m/z* 635 showed peaks at t_R = 24.45 and 26.93 min corresponding to two different trigalloyl glucose derivatives and RIC relative to *m/z* 787 showed peaks at t_R = 17.51 min corresponding to tetragalloyl glucose.

For ANP-3 fraction, RIC relative to *m/z* 441 showed one peak at t_R = 24.82 min corresponding to catechin gallate, RIC relative to *m/z* 593 showed two peaks at t_R = 29.51 and 30.34 min corresponding to two different gallocatechin digallate, RIC relative to *m/z* 745 showed one peak at t_R = 30.34 min corresponding to catechin trigallate and RIC relative to *m/z* 897 showed one peak at t_R = 31.68 min corresponding to catechin tetragallate.

This qualitative analysis provided a valuable fingerprint of the main metabolites occurring in the 80% EtOH extract of *A. nilotica* pods.

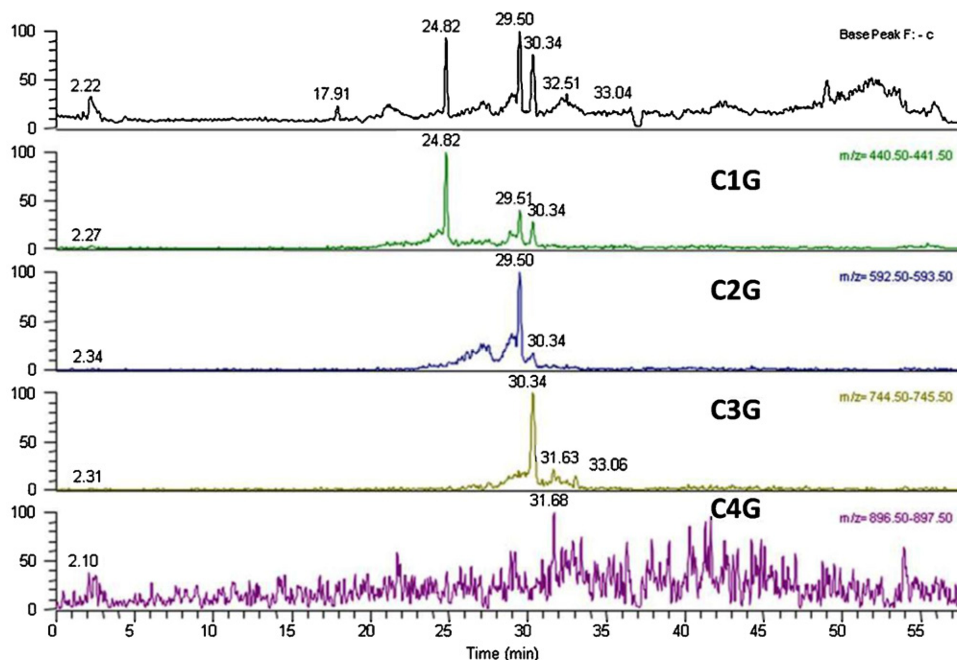

Fig. 6. LC–ESI-MS TIC (negative ion mode) (base peak in the range 250–1000 *m/z*) and RICs (reconstructed ion chromatograms) of ANP-3 fraction.

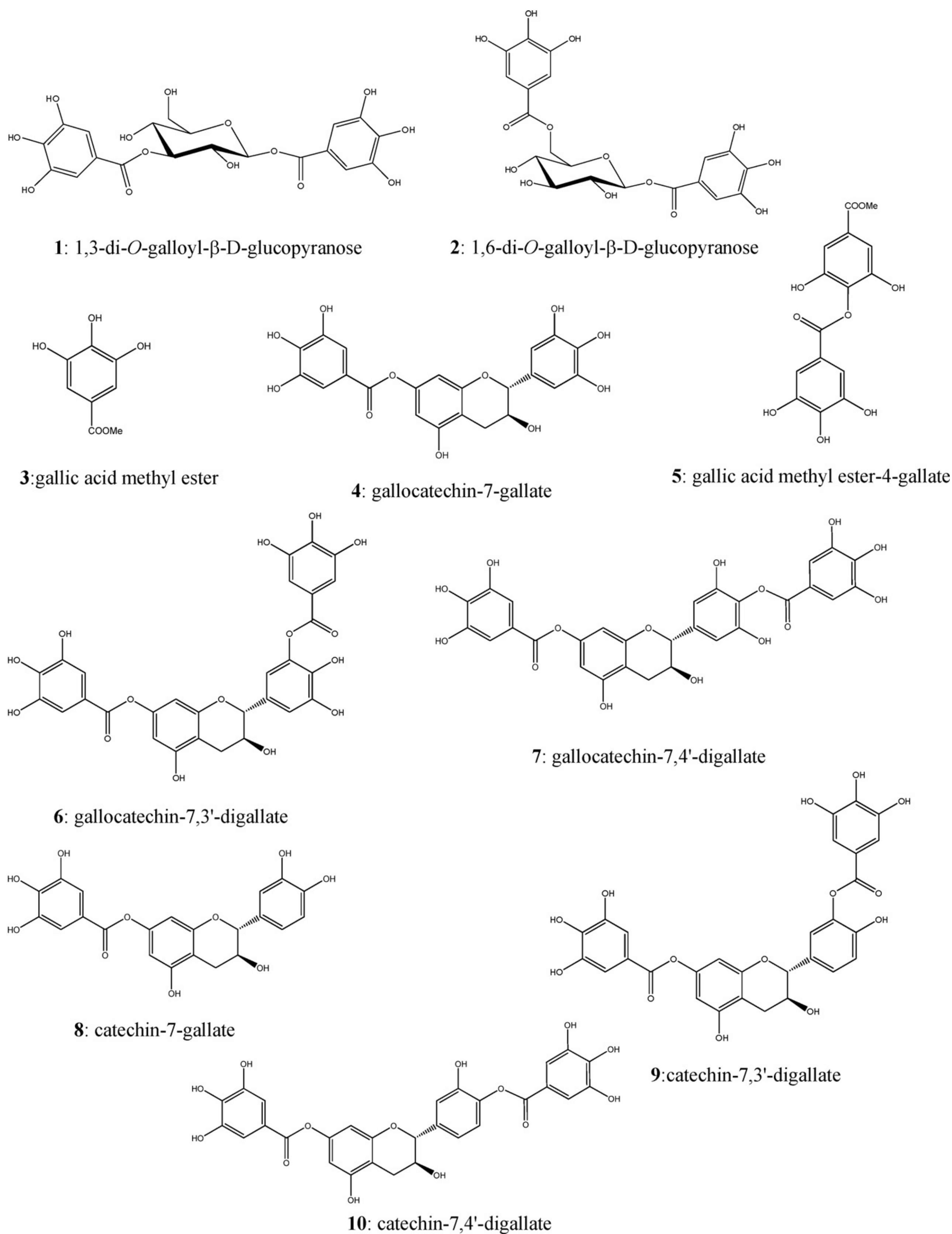

Fig. 7. Compounds **1**–**10** from *A. nilotica* pods.

With the aim to confirm the presence of compounds detected in the 80% EtOH extract of *A. nilotica* pods, a procedure of isolation was developed (see Section 2.7). The structures of the isolated compounds were elucidated by NMR and MS analysis [8,22–24]. They resulted to be 1,3-di-*O*-galloyl-β-D-glucopyranose (**1**), 1,6-di-*O*-galloyl-β-D-glucopyranose (**2**), gallic acid methyl ester (**3**), gallocatechin-7-*O*-gallate (**4**), gallic acid methyl ester-4-gallate (**5**), gallocatechin-7,3′-di-*O*-gallate (**6**), gallocatechin-7,4′-di-*O*-gallate

(**7**), catechin-7-*O*-gallate (**8**), catechin-7,3′-di-*O*-gallate (**9**) and catechin-7,4′-di-*O*-gallate (**10**). Compound **4** resulted to be a new natural compound. Compounds **5**, **6**, **7** and **1** are reported for the first time in *Acacia* genus, while compound **10** is reported for the first time in *Acacia nilotica*.

All the isolated compounds are reported in Fig. 7.

3.3. NMR characterization of compound 4

The new compound **4** exhibited the [M−H]$^-$ peak in the ESI-MS spectrum at *m/z* 457. The second order MS/MS spectrum of the ion at *m/z* 457 led to the significant fragment ion peaks at *m/z* 305 and *m/z* 289. This fragmentation pattern was characteristic of a gallocatechin gallate derivative. The ^{1}H NMR spectrum of compound **4** showed evidence of a gallocatechin core with the signals at δ_H 6.44 (2H, *s*, H-2′, H-6′), 6.24 (2H, *s*, H-6, H-8), (*s*), 4.67 (1H, *d*; *J* = 7.0 Hz, H-2), 4.08 (1H, *m*, H-3), 2.64 (1H, *dd*; *J* = 6.2, 15.8 Hz) and 2.91 (1H, *dd*; *J* = 4.5, 15.8 Hz). Furthermore, the signal at δ_H 7.19 indicated the presence of a gallic acid moiety. The HSQC experiment provided all the connectivities between protons and their respective carbons while the HMBC correlations allowed the assignment of quaternary carbons as reported in Table 1. The position of the galloyl moiety at C-7 was deduced from the ROESY correlation between the proton signal at δ_H 7.19 of the galloyl unit and the signal at δ_H 6.24 ascribable to H-6 and H-8. On the basis of these data, compound **4** was identified as the new gallocatechin-7-*O*-gallate.

3.4. TEAC results

The antioxidant activity of ANP-2 and ANP-3 samples and compounds **1**-**10** was studied in the TEAC assay [25–27]. This method measures the relative ability of antioxidant substances to scavenge the radical cation 2,2′-azino-bis-(3-ethylbenzothiozoline-6-sulfonate) (ABTS$^{\bullet+}$) as compared to a standard amount of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid), a water-soluble vitamin E analogue. The activity of the tested samples was expressed as TEAC (Trolox Equivalent Antioxidant Capacity) values; TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as a 1 mM concentration of the antioxidant investigated sample. All the tested fractions exhibited good free radical scavenging activity (Table 2). ANP-2 and ANP-3 fractions showed a very high activity; among their constituents, catechin-7,3′-di-*O*-gallate (**9**) and catechin-7,4′-di-*O*-gallate (**10**) showed the strongest scavenging activity towards ABTS radical, with TEAC values (mM) of 3.11, 3.05, respectively, followed by gallocatechin-7,3′-di-*O*-gallate and gallocatechin-7,4′-di-*O*-gallate which showed a TEAC value higher than that of quercetin, the reference antioxidant compound (Table 2).

These results demonstrate that most of the tested samples possess high activity as ABTS scavangers, showing the potential use of *A. nilotica* as a source of antioxidant principles.

Table 2
Antioxidant activity of compounds **1**–**10** and purified fractions (ANP-2 and ANP-3) in the TEAC assays.[a]

Sample	TEAC (mM) ± SD[b]	TEAC (μg/mL) ± SD[b]
Gallic acid methyl ester (**3**)	1.82 ± 0.06	–
Gallic acid methyl ester-4-gallate (**5**)	1.20 ± 0.03	–
Catechin-7-gallate (**8**)	3.00 ± 0.03	–
Gallocatechin-7-gallate (**4**)	2.06 ± 0.03	–
Catechin-7,3′-digallate (**9**)	3.11 ± 0.01	–
Catechin-7,4′-digallate (**10**)	3.05 ± 0.05	–
Gallocatechin-7,3′-digallate (**6**)	2.90 ± 0.05	–
Gallocatechin-7,4′-digallate (**7**)	2.79 ± 0.01	–
1,3-di-*O*-galloyl-β-D-glucose (**1**)	0.16 ± 0.02	–
1,6-di-*O*-galloyl-β-D-glucose (**2**)	0.91 ± 0.04	–
ANP 2	–	3.16 ± 0.07
ANP 3	–	3.78 ± 0.06
Quercetin	2.60 ± 0.02	–

[a] For protocols used, see Section 3.
[b] *n* = 3.

3.5. Quantitative HPLC–ESI-QqQ-MS/MS analysis of *A. nilotica* extract

An LC–ESI-MS/MS method in the multiple reaction monitoring (MRM) mode was developed for the quantitative determination of the polyphenolic constituents occurring in *A. nilotica* pods.

For quantitative determination compounds peculiar of this species were selected, and in particular, compounds **3** and **5** were not quantified because they are not characteristic of *Acacia nilotica*.

For this purpose a mass spectrometer equipped with a triple quadrupole analyser was employed.

In order to monitor the compounds **4**, **6**–**10** by MRM, the specific fragmentation reaction [M−H]$^-$ → [M−H−152]$^-$ was selected. For these compounds the loss of the galloyl moiety was the dominant fragmentation. Compound **1** was monitored by the transition from the specific deprotonated molecular ion [M−H]$^-$ to the corresponding fragment ion [M−H−170]$^-$, relative to the loss of gallic acid and water, while compound **2** was monitored by the transition from the specific deprotonated molecular ion [M−H]$^-$ to the corresponding fragment ion [M−H−212]$^-$. Fig. 8 shows negative ESI-MS/MS product ion spectra of deprotonated molecular ions of compound **1** (Fig. 8A) and compound **2** (Fig. 8B) and their major fragmentations chosen for MRM analysis, that permit to use different transitions for the two compounds having the same molecular weight and differing only in the position of the gallic acid on the sugar.

MRM analyses of *A. nilotica* extract were made according to the experimental procedure described in Section 2.

The calibration curves obtained by plotting the area ratios between the external standards versus known concentrations of compounds were linear in the range of 1–100 μg/mL.

Table 3 shows the results of the quantitative analysis; it is possible to observe that the main compounds are gallocatechin-7,3′-digallate (**6**) and gallocatechin-7,4′-digallate (**7**), followed by gallocatechin-7-*O*-gallate (**4**), while the other compounds occur in smaller quantities. The analytical method has proven to be reliable with a good reproducibility, attested by the low values of standard deviation (Table 4).

3.6. Validation of the method

The HPLC–MS/MS assay was validated according to the European Medicines Agency (EMEA) guidelines relating to the validation of analytical methods [28]. The method based on the characteristic fragmentation reactions of polyphenols was highly specific with no other peaks interfering at the retention times of the marker compounds in the MRM chromatograms. The intra-day accuracy and precision were calculated by analysing three samples of catechin-7-*O*-gallate at three different concentration levels, namely, 1, 10 and 50 μg/mL, on the same day. Inter-day estimates were performed over three consecutive days. The standard deviation was <5%. The limit of quantification (LOQ), defined as the lowest concentration of compound quantifiable with acceptable accuracy and precision, was determined by injection of a series of diluted standard solutions until a signal-to-noise ratio of 10 was attained. The LOQ values calculated for each compound were less than 15 ng/mL. A recovery

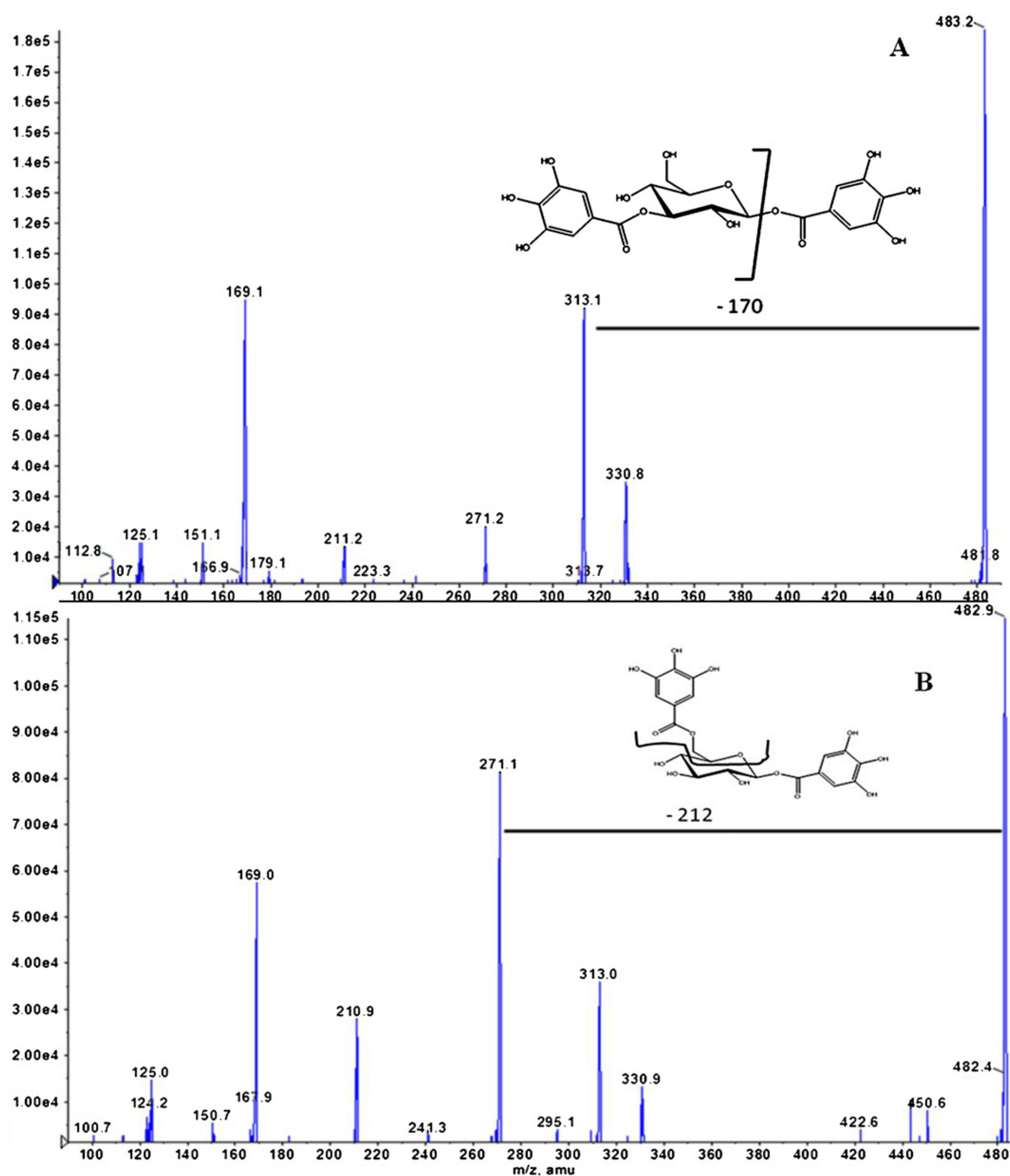

Fig. 8. Negative ESI-QqQ-MS/MS spectra of compound **1** (A) and **2** (B).

Table 3
Calibration curve data for the analysis of compounds **1**, **2**, **4**, **6**–**10**.

Compounds	t_R	Precursor ion [M−H]⁻	Product ion [A−H]⁻	Calibration curve equation	r^2
1,3-Di-*O*-galloyl-β-D-glucopyranose (**1**)	14.9	483	313	$y = 186x - 230$	0.999
1,6-Di-*O*-galloyl-β-D-glucopyranose (**2**)	17.2	483	271	$y = 165x + 346$	0.999
Catechin-7,3′-di-*O*-gallate (**9**)	24.6	593	441	$y = 633x - 70.6$	0.999
Catechin-7,4′-di-*O*-gallate (**10**)	24.9	593	441	$y = 599x - 54.1$	0.995
Catechin-7-*O*-gallate (**8**)	23.6	441	289	$y = 2.91e + 3x + 598$	1.000
Gallocatechin-7,3′-di-*O*-gallate (**6**)	23.0	609	457	$y = 30.9x - 46.9$	0.999
Gallocatechin-7,4′-di-*O*-gallate (**7**)	23.7	609	457	$y = 28.7x - 36.2$	0.997
Gallocatechin-7-*O*-gallate (**4**)	21.3	457	305	$y = 300x + 350$	0.999

Table 4
Quantitative results by MRM LC/ESI/MS/MS.

Compounds	LOQ (ng/mL)	LOD (ng/mL)	Dried pods concentration ($\mu g\,g^{-1}$ (SD))
1,3-Di-*O*-galloyl-β-D-glucopyranose (**1**)	5.23	1.21	3.13 (0.36)
1,6-Di-*O*-galloyl-β-D-glucosepyranose (**2**)	5.14	1.05	20.47 (2.45)
Catechin-7,3′-di-*O*-gallate (**9**)	6.32	1.23	16.13 (3.03)
Catechin-7,4′-di-*O*-gallate (**10**)	6.11	1.52	20.41 (3.11)
Catechin-7-*O*-gallate (**8**)	5.98	1.11	26.47 (1.44)
Gallocatechin-7,3′-di-*O*-gallate (**6**)	5.74	1.08	222.01 (14.14)
Gallocatechin-7,4′-di-*O*-gallate (**7**)	5.55	1.11	203.02 (8.11)
Gallocatechin-7-*O*-gallate (**4**)	3.98	1.07	84.42 (5.16)

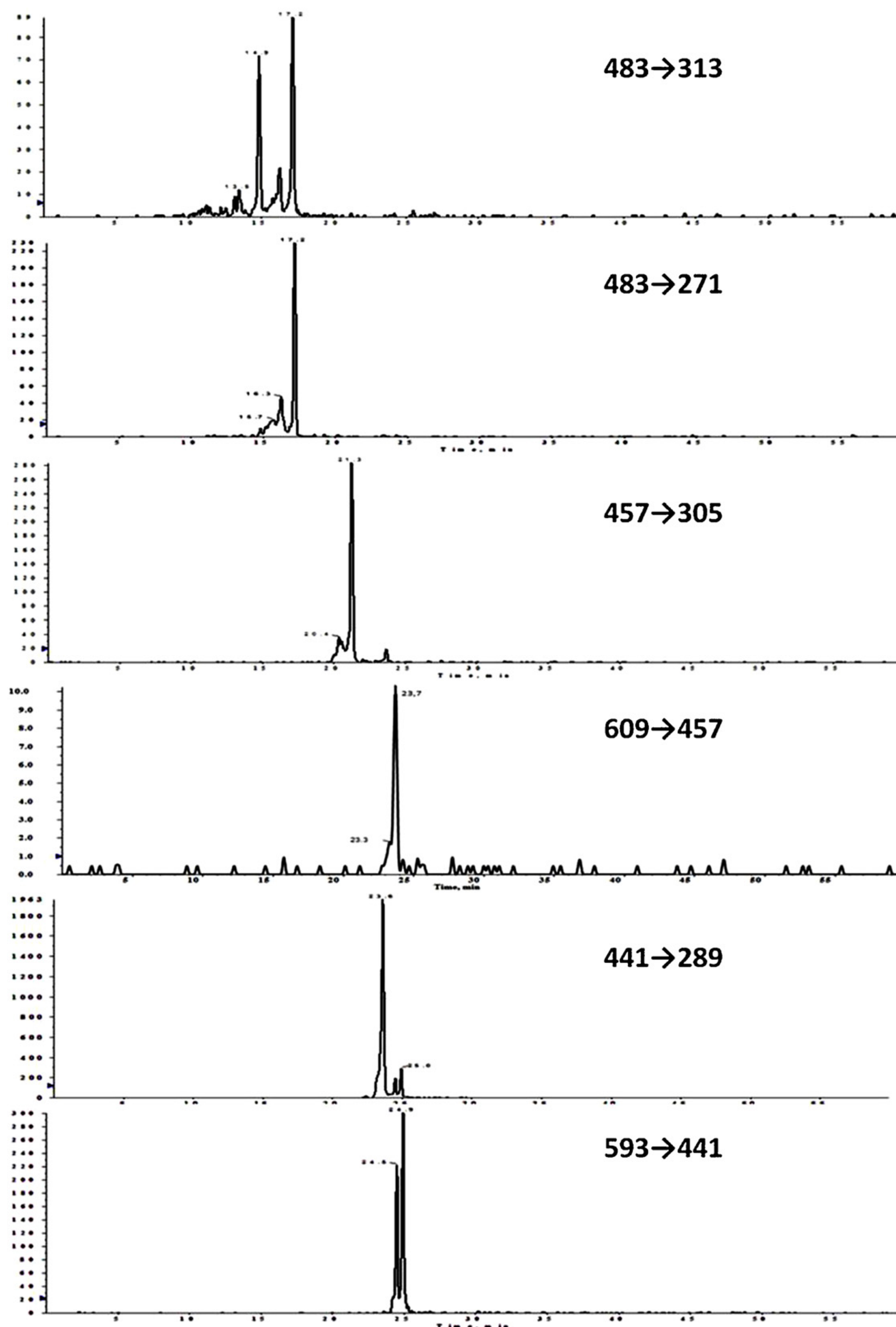

Fig. 9. LC/ESI(QqQ)/MS/MS XICs (extracted ion chromatograms) of MRM analysis of compounds **1**, **2**, **4** and **6**–**10**.

study was performed by adding an accurately known amount of the corresponding marker compounds at three concentration levels (1, 5 and 50 $\mu g\,g^{-1}$ of plant), to a sample of *A. nilotica* pods that had previously been analysed. Recovery was between 96.82% and 102.47% with standard deviations values of less than 2.75% for all the seven compounds (Fig. 9).

4. Conclusions

Acacia nilotica is a rich source of polyphenolic constituents, which contains different galloylated derivatives of catechin and gallocatechin. These compounds are hypothesized to possess antioxidant properties and to play a role in the anti-inflammatory

activity of several plants. The strong free radical scavenging activity exhibited by most of these compounds in the TEAC assay is in agreement with this statement.

An LC–MS/MS MRM method was developed and was successfully applied to the quantitative determination of the phenolic constituents occurring in *A. nilotica* pods. This method resulted to be straightforward and convenient requiring a very fast sample preparation procedure; thus it can easily be applied to other catechin producing plants to quantify these bioactive compounds in raw material and final products.

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