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Phenolic compounds from *Byrsonima crassifolia* L. bark: Phytochemical investigation and quantitative analysis by LC-ESI MS/MS

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ABSTRACT

Phytochemical investigation of the methanolic extract of *Byrsonima crassifolia*'s bark led to the isolation of 8 known phenolic compounds 5-*O*-galloylquinic acid, 3-*O*-galloylquinic acid, 3,4-di-*O*-galloylquinic acid, 3,5-di-*O*-galloylquinic acid, 3,4,5-tri-*O*-galloylquinic acid, (+)-epicatechin-3-gallate along with (+)-catechin and (+)-epicatechin.

Due to their biological value, in the present study, a high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, working in multiple reaction monitoring (MRM) mode, has been developed to quantify these compounds. *B. crassifolia* bark resulted in a rich source of phenolic compounds and particularly of galloyl derivates. The proposed analytical method is promising to be applied to other galloyl derivatives to quantify these bioactive compounds in raw material and final products.

1. Introduction

Byrsonima crassifolia L. (Malpighiaceae) is a small evergreen tree and its bark is employed in folk medicine to treat coughs, gastrointestinal disorders, gynaecological inflammations, skin infections [1,2] and snakebites [3]. Experimental studies on this species showed antioxidant properties for the hydroalcoholic extracts of its bark and leaves [4], spasmogenic effects [5,6], antimicrobial activity for roots and stems organic extracts [7–9], antiprotozoarial activities for bark and leaves alcohol extracts [10,11], and central nervous system depressant activity for leaves and bark aqueous extracts [12]. Recently, topical anti-inflammatory activity was demonstrated for its barks extracts [13]. No phytochemical studies were previously reported on the bark of *B. crassifolia*.

Byrsonima species are reported to be rich in galloylquinic acids as well as flavonoids, triterpenes, steroids, sulphonoglycolipids, aromatic esters, proanthocyanidins and catechins [14,15].

Phenolic compounds are natural antioxidants and could play a preventive role in the development of cancer and heart disease [16]. A number of biological and pharmacological activities have also been reported for these compounds, including free radicals scavenging, apoptosis of cancer cells [17–19], antiherpectic, anti-human immunodeficiency virus (HIV) reverse transcriptase and anti-HIV activity [20–23]. In addition, galloylquinic acid derivatives have shown high activity against bronchial hyper-reactivity and allergic reactions [23].

Important sources of galloylquinic acids are green and black tea, tannin and genera as *Castanopsis*, *Quercus* and *Byrsonima*, where they may contribute to the pharmacological properties of derived herbal preparations [24].

High performance liquid chromatography/tandem mass spectrometry (LC–MS^n) has been successfully used for characterizing and identifying galloyl derivatives in complex samples [15]. In the last decade LC/ESI–MS/MS has become one of the major tools for biological and chemical analyses for quantitative purposes [25]. LC–MS/MS techniques provide specific, selective and sensitive quantitative results often with reduced sample preparation and analysis time if compared to other commonly employed techniques. In consideration of the biological interest of the compounds identified in the present study, and in particular the galloyl quinic derivatives (**1**–**6**) a high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, working in multiple reaction monitoring (MRM) mode, has been developed to quantify these compounds.

2. Materials and methods

2.1. Chemicals

Solvents used for extraction were of high purity and purchased from Carlo Erba (Milano, Italy). HPLC grade methanol (MeOH), acetonitrile (ACN), formic acid (HCOOH) and trifluoroacetic acid (TFA) were purchased from Merck (Merck KGaA, Darmstadt, Germany). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Chlorogenic acid standard was purchased from Extrasynthese

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(Geney, France). All other chemicals used in this study were of analytical grade.

2.2. Plant material

Plant materials were collected in Belize (Central America) and authenticated by Professor M.J. Balick. Voucher specimens (no. 26) were dried and deposited at the New York Botanical Garden (NY, USA).

2.3. Preparative chromatography

An Agilent (Palo Alto, CA, USA) 1100 series chromatographic system, comprising a G-1312 binary pump, a G-1328A Rheodyne injector, a G-1322A degasser and a G-1315A photodiode array detector, was employed. Analyses were carried out using a dC18 Atlantis 250 mm × 10 mm (Waters, Milford, MA, USA) and C18 Onyx Monolithic 100 mm × 4.6 mm (Phenomenex, USA) columns. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

2.4. Extraction and isolation procedures

The air-dried and powdered plant material (174.52 g of *B. crassifolia* bark) was sequentially extracted, two times for three days, with 1 l of petroleum ether, chloroform and methanol, at room temperature. The solvents were removed from the filtered extract solutions under vacuum at 30 °C in a rotary evaporator, until dry petroleum ether, chloroform and methanol extracts were obtained (2.24 g, 0.5 g and 22.06, respectively). Part of the methanol extract (3 g) was fractionated initially on a 100 cm × 5.0 cm Sephadex LH-20 column, using CH_3OH as mobile phase, and 120 fractions (8 ml each) were obtained. Fractions 25–27 (sample **a**) (26.3 mg), 28–32 (sample **b**) (86.7 mg), 33–35 (sample **c**) (21.9 mg), 36–40 (sample **d**) (33.3 mg), 41–45 (sample **e**) (21.7 mg) and 62-63 (sample **f**) (52 mg) were chromatographed by HPLC–UV using a dC18 Atlantis column for fractions **a**–**e** and a C18 Onyx Monolithic column for fraction **f** at flow rate of 2 ml/min. Mobile phase employed was represented by mixtures of water containing 0.05% trifluoroacetic acid (TFA; solvent A) and acetonitrile containing 0.05% TFA (solvent B). For samples **a**–**e**, elution was with a gradient commencing at 100% A and changing to 80:20 (A:B) in 15 min, then from 80:20 (A:B) to 60:40 (A:B) in 20 min and finally to 100% B in 15 min. For sample **f** elution was with a gradient commencing at 100% A and changing to 90:10 (A:B) in 7 min and finally from 90:10 (A:B) to 70:30 (A:B) in 40 min. The detection wave-lengths selected were 210 nm, 254 nm and 350 nm.

From sample **b** compound **1** (6.1 mg, t_R = 14), from sample **c** compound **2** (2 mg, t_R = 14.7), from sample **d** compounds **3** (1.1 mg, t_R = 20.5), **4** (1.3 mg, t_R = 23.4), **7** (1.7 mg, t_R = 26.3) and **8** (2.1 mg, t_R = 29.2), from sample **e** compound **5** (3.9 mg, t_R = 27.4), from sample **f** compound **6** (2.2 mg, t_R = 18.2) were obtained.

Optical rotations were measured on a JASCO DIP 1000 polarimeter.

(+)-epicatechin-3-gallate (5): red powder; $[\alpha]^{25}_D$ +136.9 (*c* 0.091, MeOH); UV (MeOH) λ_{max} 280 nm,
(+)-epicatechin (7): red powder; $[\alpha]^{25}_D$ +57.2 (*c* 0.166, MeOH); UV (MeOH) λ_{max} 280 nm,

2.5. NMR analysis

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

All the isolated compounds were isolated and characterized by NMR and MS data in comparison with those reported in literature [16,24,26–28].

2.6. ESI/MS and ESI/MS/MS analyses

The electrospray ionisation (ESI) source of a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was tuned with a standard solution of 3,4,5-tri-*O*-galloylquinic acid (**5**) in methanol (l μg/ml) infused at a flow rate of 10 μl/min with a syringe pump. The mass spectrometer was operated in the negative ion mode with a capillary voltage of −7 V, spray voltage of 5 kV, tube lens offset of 10 V, capillary temperature of 280 °C, and sheath gas (nitrogen) flow rate of 0 (arbitrary units).

ESI/MS data for 3,4,5-tri-*O*-galloylquinic acid (**5**) were acquired in a similar manner using an Applied Biosystems (Foster City, CA, USA) API2000 triple quadrupole instrument. In this case the mass spectrometer was operated in the negative ion mode with optimised conditions: declustering potential −57.6 eV, focusing potential −66.8 eV, entrance potential −7.6 eV. In all cases, the MS spectra were acquired and elaborated using the software provided by the manufacturer.

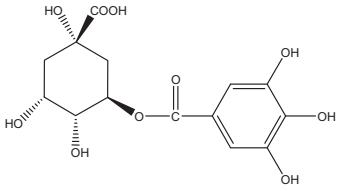
2.7. LC-ESI–MS and LC-ESI–MS/MS analyses

Qualitative LC-ESI/MS was performed using a Thermo Finnigan SpectraSystem HPLC equipped with a Waters (Milford, MA, USA) Atlantis T3 C18 column (150 mm × 2.1 mm i.d., 5 μm d) and coupled to a LCQ Deca ion trap. Linear gradient elution with a mobile phase comprising water acidified with 0.05% formic acid (solvent **A**) and acetonitrile acidified with 0.05% formic acid (solvent **B**) commenced at 100:0 (A:B) and changed to 80:20 (A:B) in 15 min, then from 80:20 (A:B) to 60:40 (A:B) in 20 min and finally from 60:40 (A:B) to 100% B in 15 min. The flow (200 μl/min) from the chromatograph was inject directly into the ESI source, maintained at a temperature of 280 °C and MS were measured under the optimised parameters indicated for ESI–MS with nitrogen supplied at a flow rate of 50 (arbitrary units).

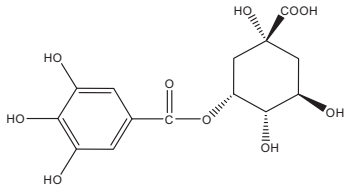
Quantitative analyses were performed on an Agilent (Palo Alto, CA, USA) 1100 HPLC system equipped with a Waters Atlantis T3 C18 column (150 mm× 2.1 mm i.d., 5 μm) and coupled to an Applied Biosystems API2000 triple quadrupole instrument. A gradient elution was performed by using a mobile phase A represented by water acidified with HCOOH (0.05%) and a mobile phase B represented by acetonitrile acidified with HCOOH (0.05%). The gradient started from 0% of eluent B to achieve the 20% of solvent B in 15 min and finally reached 40% of solvent B in 25 min. The flow (200 μl/min) generated by chromatographic separation was directly injected into the electrospray ion source.

MS spectra were acquired in negative ion mode and elaborated using the software provided by the manufacturer, and reconstructed ion chromatograms (RICs) were elaborated in order to identify galloylated compounds from their deprotonated molecular ions.

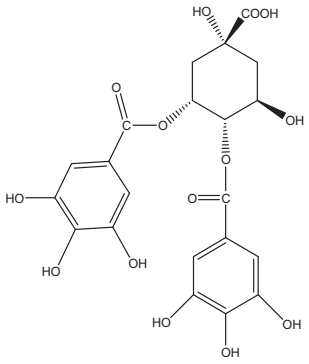
API2000 ESI source was tuned with a 3,4,5-tri-*O*-galloylquinic acid (**5**) standard solution in methanol (l μg/ml) infused at the flow rate of 10 μl/min with a syringe pump. The mass spectrometer was operated in the negative ion mode under the following conditions: declustering potential of −57.9 eV, focusing potential of −66 eV, entrance potential −7.6 eV, collision energy −30 eV, collision cell exit potential −15 eV, ion spray voltage −4200, temperature 300 °C. The instrument was used in the tandem MS mode, multiple reaction monitoring (MRM). Frag-



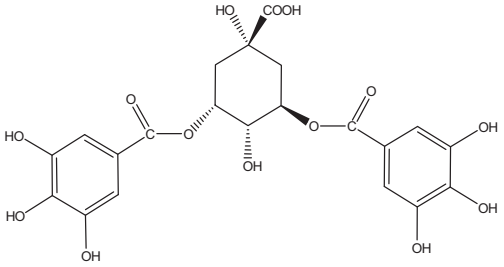
1: 5-*O*-galloylquinic acid



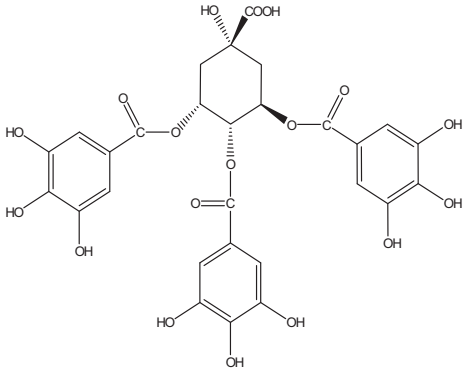
2: 3-*O*-galloylquinic acid



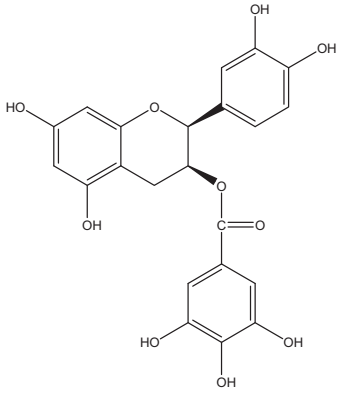
3: 3,4-di-*O*-galloylquinic acid



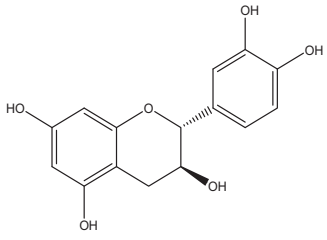
4: 3,5-di-*O*-galloylquinic acid



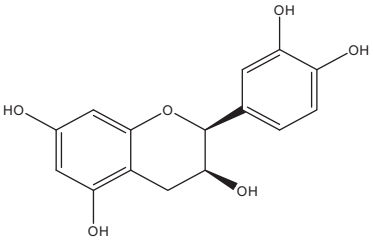
5: 3,4,5-tri-*O*-galloylquinic acid



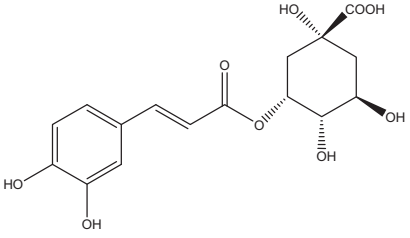
6: (+)-epicatechin-3-gallate



7: (+)-catechin



8: (+)-epicatechin



IS: 3-*O*-caffeoylquinic acid

Fig. 1. Compounds isolated form methanolic extract of *Byrsonima crassifolia*'s bark.

mentation reactions selected for each compound are described below.

2.8. Calibration and quantification

In order to prepare the specific calibration plot for each compound under investigation, stock solutions of standard **1**–**6** (1 mg/ml) were prepared by dissolving each compound in MeOH. The resulting stock solution was diluted with methanol in order to obtain reference solutions containing 25, 40, 50, 60, 75, 100, 200 and 300 μg/ml of external standard. An appropriate amount of the internal standard (IS; chlorogenic acid) was added to each reference standard solution to give a final concentration of 5 μg/ml. Calibration curves were constructed by analysing reference Standard/IS solutions in triplicate at each concentration level. The ratios of the peak areas of the external standard to those of the IS were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves. All

quantitative data were elaborated with the aid of Analyst software (Applied Biosystems).

2.9. Sample preparation

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

For quantitative purpose a solution 3 mg/ml of methanolic extract, and a volume of 10 μl was injected in the chromatographic system.

3. Results and discussion

3.1. Phytochemical investigation

Phytochemical investigation of the methanolic extract of the bark of *B. crassifolia* led to the isolation of 6 galloylated derivatives such as 5-*O*-galloylquinic acid (**1**), 3-*O*-galloylquinic acid (**2**), 3,4-di-*O*-galloylquinic acid (**3**), 3,5-di-*O*-galloylquinic acid (**4**), 3,4,5-tri-*O*-galloylquinic acid (**5**), (+)-epicatechin-3-gallate (**6**) along with (+)-catechin (**7**) and (+)-epicatechin (**8**).

All the isolated compounds where characterized by NMR, MS and physical data in comparison with those reported in literature. The eight isolated compounds are reported, with the addition of internal standard discussed below in Fig. 1. With the exception of compounds **7** and **8**, all of them are galloylated derivatives.

In addition, is to be noted that the (+) epicatechin, a relatively less common enantiomer of (−) epicatechin, is reported in *Byrsonima* species. Recently the presence of the (+) enantiomer of epicatechin was described in *Byrsonima* species, by using chiral chromatography by Rinaldo et al. [29]. In the present work NMR and physical data confirmed the presence of the (+) epicatechin and (+) epicatechin gallate in *B. crassifolia* bark's extract.

3.2. Qualitative LC–MS analysis

In order to realise a qualitative analysis on the phenol derivatives in *B. crassifolia* barks extract MS experiments were performed by using an LC–MS system equipped with an ESI source and an Ion Trap analyser. Many gradients based on MeOH/water and MeCN/water were tested as mobile phases, together with different RP18 columns for analytical HPLC separations. The best result was obtained using the Atlantis T3 C18 column and a gradient of MeCN/water, as described in the Experimental. Separations using 0.1% formic acid in the mobile phase gave increased retention, sharper peaks and considerable improvement in signal-to-noise ratio than without the addition of the formic acid.

HPLC-ESI-ITMS has become a very powerful tool for the analysis of phenolic compounds commonly found in complex mixtures, rendering it one of the most attractive techniques for on-line investigations of plant extracts. For HPLC–MS analyses, negative ion mode was selected allowing us to obtain high sensitivity and best results to identify galloylated compounds.

A full MS scan (150–1000), in the form of a total ion current (TIC) chromatogram, was initially acquired, following which RICs were derived for each of the expected *m*/*z* values based on the molecular weights of the isolated compounds (Fig. 2). The components were efficiently separated, and it was possible to recognize peaks corresponding to the deprotonated molecular ions of various galloylated derivatives. On the basis of their *m*/*z* values in the TIC profile, RICs were generated for specific compounds expected to be present in the sample and the following constituents were identified: **1** and **2** (tR 9.39 and 10.31, *m*/*z* 343), **3** and **4** (tR 14.20 and 14.59, *m*/*z* 495), **5** (tR 16.90, *m*/*z* 647), **6** (tR 21.27, *m*/*z* 441).

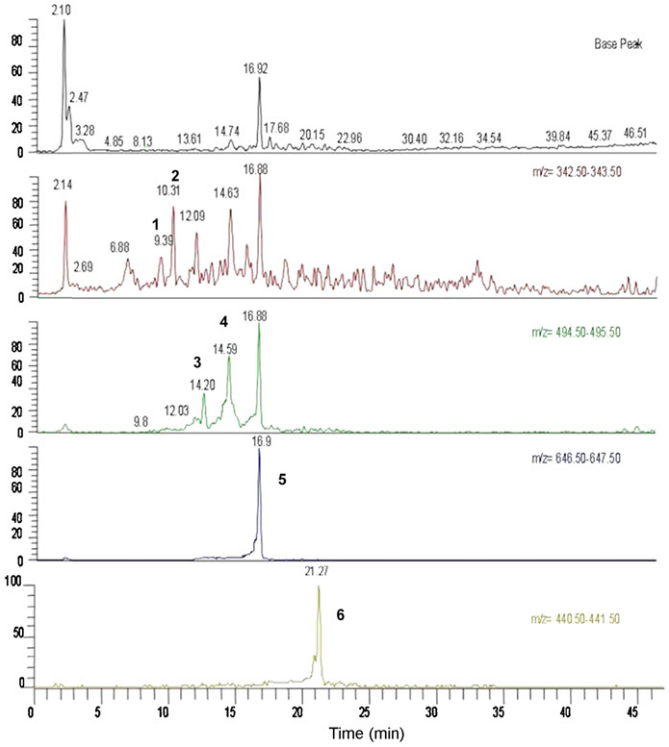


Fig. 2. Qualitative metabolite fingerprint of methanolic extract of *Byrsonima crassifolia*'s bark showing the TIC profile (top panel) and various RICs (lower panels) obtained from positive ion mode HPLC-ESI-IT-MS analysis.

This form of qualitative analysis provides a valuable fingerprint of the major metabolites present in bark methanolic extract of *B. crassifolia*. Detailed HPLC analyses revealed the presence of selected components.

3.3. Quantitative analysis of compounds *1*–*6* in B. crassifolia bark by LC-ESI(QqQ)/MS/MS

Several studies are present in literature reporting the use of liquid chromatography in-space tandem mass spectrometry to perform quantitative analyses with better accuracy [30,31]. Thus, for quantitative purpose, an accurate method on a mass spectrometer equipped with a triple quadrupole analyzer was developed for the analyses of compounds **1**–**6**. Since it is important to find a specific fragment for each analyte, ESI–MS/MS analyses were recorded and fragmentation patterns studied for the six standard compounds. However, for compounds **1**–**6**, which are galloylated derivatives, the loss of gallic acid unit was the predominant fragmentation. Thus, the transition from specific deprotonated molecular ion $[M-H]^-$ to the corresponding fragment ion $[M-H-152]^-$ for each standard was selected in order to monitor these galloylated compounds. Under the conditions of HPLC–ESI–MS/MS, IS (internal standard, chlorogenic acid) was characterized by MRM through the transition from precursor ion *m*/*z* 353.0 to product ion *m*/*z* 191.0. The calibration curves were constructed by plotting the area ratio between the external standards and internal standard against the known concentration of each compound, and were found to be linear in the range of 25–300 μg/ml. Five aliquots of each extract of *B. crassifolia* were analysed in order to quantify the content of compounds **1**–**6**. Table 1 reports quantitative analysis results (Fig. 3).

3.4. Validation

The HPLC–MS/MS analytical method was validated according to the European Medicines Agency (EMEA) guidelines relating to

Table 1
Calibration curve data for the analysis of compounds **1**–**6**.

Compound	Precursor ion $[M-H]^-$	Product ion $[A-H]^-$	r^2	Calibration curve equation
5-*O*-galloylquinic acid (**1**)	343	191	0.998	$y = 0.000107 \times -0.0031$
3-*O*-galloylquinic acid (**2**)	343	191	0.995	$y = 4.02e^{-005} \times -0.0005$
3,4-di-*O*-galloylquinic acid (**3**)	495	343	0.999	$y = 0.00036 \times +0.0091$
3,5-di-*O*-galloylquinic acid (**4**)	495	343	0.997	$y = 0.00079 \times -0.0115$
3,4,5-tri-*O*-galloylquinic acid (**5**)	647	495	0.999	$y = 0.01350 \times +0.1120$
(+)-epicatechin-3-gallate (**6**)	441	289	0.992	$y = 0.00055 \times -0.0826$

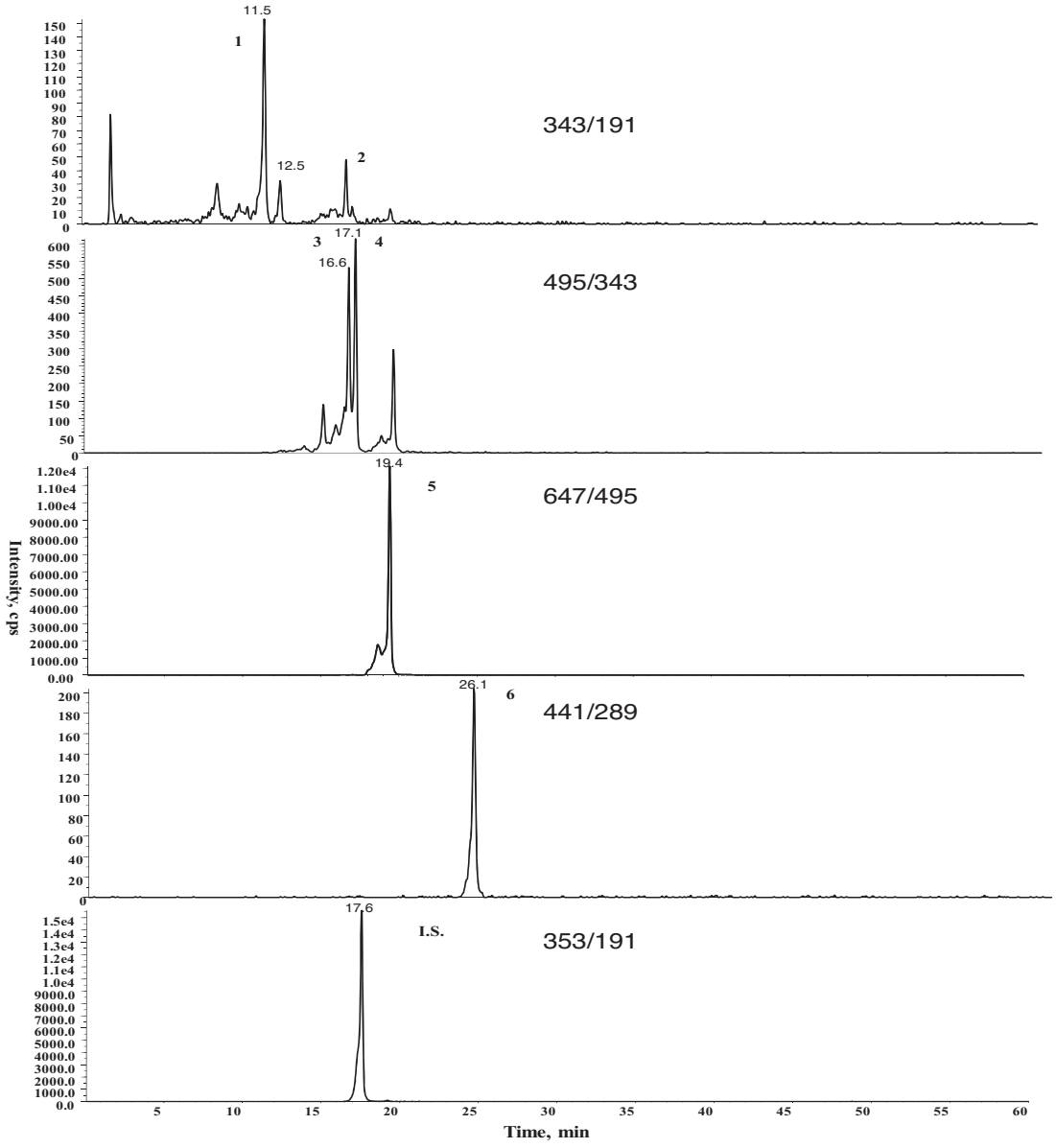


Fig. 3. Negative ion mode LC-ESIMS/MS MRM analyses of *Byrsonima crassifolia*'s bark's methanolic extract. Profiles of the specific transition from parent ions to product ions in MRM mode for compounds **1**–**6** and internal standards.

Table 2
Quantitative results for compounds **1**–**6** by MRM LC/ESI/MS/MS.

Compound	LOQ (ng/ml)	LOD (ng/ml)	Dried barks concentration (mg/g) (S.D.)
5-*O*-galloylquinic acid (**1**)	14.11	1.15	11.29 (0.73)
3-*O*-galloylquinic acid (**2**)	18.52	2.23	14.79 (0.74)
3,4-di-*O*-galloylquinic acid (**3**)	16.35	1.52	5.59 (0.46)
3,5-di-*O*-galloylquinic acid (**4**)	11.42	1.45	3.85 (0.36)
3,4,5-tri-*O*-galloylquinic acid (**5**)	19.14	1.68	1.31 (0.06)
(+)-epicatechin-3-gallate (**6**)	17.01	2.21	12.53 (1.06)

the validation of analytical methods [32]. The method based on the characteristic fragmentation reactions of phenolic compounds was highly specific with no any other peak interfering at the retention times of the marker compounds (compounds **1**–**6**) in the MRM chromatograms. The intra-day accuracy and precision were calculated by analysing three samples of compound **1** at three different concentration levels, namely, 25, 50 and 300 μg/ml, on the same day. Inter-day estimates were performed over three consecutive days. The standard deviation was <5%. The calibration graphs, obtained by plotting the area obtained from external standard against the known concentration of external standard (for each compound) was linear in the range of 25–300 μg/ml. 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 the six compounds under investigation were less than 20 ng/ml. Table 2 reports validation data of the method developed for quantitative analysis of compounds **1**–**6**.

4. Conclusions

Compounds **1**–**8** are here reported for the first time in *B. crassifolia*. In addition, the presence of unusual (+) epicatechin and its 3-*O*-galloyl derivative in the bark of this species has been confirmed, as already reported in other species of the genus *Byrsonima*.

A new LC–MS/MS method was developed, for the quantitative determination of galloyl acid derivatives in the methanolic extract of *B. crassifolia* bark.

The proposed method is straightforward and convenient requiring no sample preparation procedure, short analysis time, a low flow rate and low amounts of chemicals to provide higher chromatographic resolutions. Thus this method is promising to be applied to other galloyl derivatives in plants to quantify these bioactive compounds in raw material and final products.

B. crassifolia bark resulted a rich source of phenolic compounds and particularly of galloyl derivates.

Quantitative analyses showed compounds **2** (3-O galloylchinic acid) and **6**((+) epicatechin gallate) the major phenolic constituents in the bark of *B. crassifolia*.

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