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Original Research Article

Identification and characterization of phenolics and terpenoids from ethanolic extracts of *Phyllanthus* species by HPLC-ESI-QTOF-MS/MS

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ABSTRACT

Phyllanthus species plants are a rich source of phenolics and widely used due to their medicinal properties. A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed using high-pressure liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-ESI-QTOF-MS/MS) for the identification and characterization of quercetin, kaempferol, ellagic acid and their derivatives in ethanolic extracts of *Phyllanthus* species. The chromatographic separation was carried out on Thermo Betasil C_8 column (250 mm×4.5 mm, 5 μm) using 0.1% formic acid in water and 0.1% formic acid in methanol as the mobile phase. The identification of diagnostic fragment ions and optimization of collision energies were carried out using 21 reference standards. Totally 51 compounds were identified which include 21 compounds identified and characterized unambiguously by comparison with their authentic standards and the remaining 30 were tentatively identified and characterized in ethanolic extracts of *P. emblica*, *P. fraternus*, *P. amarus* and *P. niruri*.

1. Introduction

Phyllanthus species (Euphorbiaceae) is widely distributed throughout the tropical and subtropical countries of Africa, Asia, South America and West Indies. The plants of genus *Phyllanthus* such as *P. emblica*, *P. fraternus*, *P. amarus* and *P. niruri* are extensively used in Indian System of Medicine (Ayurveda and Siddha) and Traditional Chinese Medicine due to their medicinal properties for the treatment of jaundice, asthma, malaria, eczema, wart, diarrhea and headache [1–5]. The extracts of *Phyllanthus* species have been reported to show several biological activities such as antioxidant, hepatoprotective, hypotensive, analgesic, antihepatotoxic, antiviral, antimicrobial, anticancer, anti-amnesic, antiulcer, analgesic, antiinflammatory, antiallodynic, and anti-HIV/AIDS ones [6–17]. Genus *Phyllanthus* is a rich source of phenolics and also contains alkaloids and terpenoids [14]. Phenolics can act as protective agents, inhibitors, natural animal toxicants and pesticides against invading organisms such as herbivores, nematodes, phytophagous insects, and fungal and bacterial pathogens. Phenolics are also important elements in the flavor of wine and dietary supplements due to their potent antioxidant activity [18].

Most of the qualitative and quantitative analyses of phenolics are commonly reported by traditional methods such as high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) in *Phyllanthus* species [17,19–27]. There are few reports on the comparative identification and characterization of compounds in crude extracts of *Phyllanthus* species by liquid chromatography–mass spectrometry (LC–MS) [16,17,28–35], gas chromatography–mass spectrometry (GC–MS) [36] and high performance liquid chromatography-solid phase extraction-nuclear magnetic resonance (HPLC-SPE-NMR) [37–39]. Published LC–MS methods either had very long run time [30,35] and identified few compounds with unit mass [29], or targeted or studied only one species [31–33].

The aim of this study was to develop an LC–MS/MS method for identification, characterization and distribution of phenolics and terpenoids in ethanolic extracts of *P. emblica, P. fraternus, P. amarus* and *P. niruri* using high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS/MS).

2. Experimental

2.1. Chemicals and reagents

Standards quinic acid (1), caffeic acid (2), gallic acid (4), vanillic acid (5), catechin (7), epicatechin (9), ferulic acid (13), chrysin (15),

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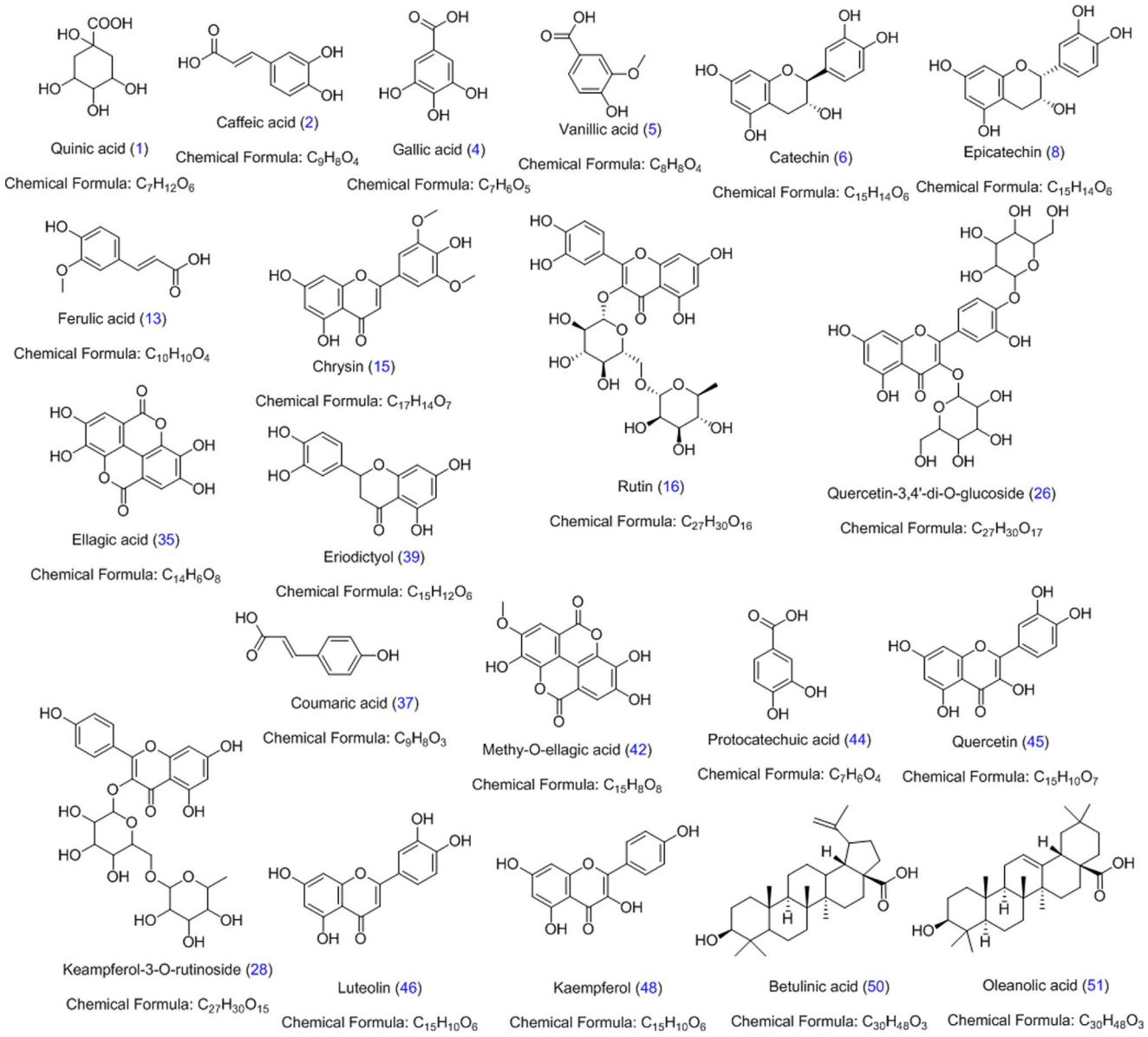

Fig. 1. Chemical structures of standard compounds.

rutin (16), quercetin-3,4′-di-*O*-glucoside (26), kaempferol-3-*O*-rutinoside (28), ellagic acid (35), coumaric acid (37), eriodictyol (39), methy-*O*-ellagic acid (42), protocatechuic acid (44), quercetin (45), luteolin (46), kaempferol (48) betulinic acid (50) and oleanolic acid (51) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1). LC–MS grade solvents (acetonitrile methanol and formic acid) were also purchased from Sigma–Aldrich (St. Louis, MO, USA) and used throughout the study. Ultra-pure water was produced by Milli-Q Advantage system (Millipore, Milford, MA, USA). AR grade ethanol (Merck, Darmstadt, Germany) was used in the preparation of the ethanolic extracts.

2.2. Plant materials

The plant parts of *P. emblica* (leaf, bark and fruit) were obtained from the campus of CSIR-Indian Institute of Integrated Medicine (CSIR-IIIM), Jammu, India, and its voucher specimen (*P. emblica*-IIIM 52949) was deposited in Biodiversity and Applied Botany Division, CSIR-IIIM, Jammu. *P. fraternus* (leaf, bark and twigs) was collected from Aizawl, Mizoram, India, and voucher specimen (*P. fraternus*-MZU/BT/18) was deposited in Department of Forestry, Mizoram University. Certified whole plants of *P. niruri* (Batch No. 10PN-1442) and *P. amarus* (Reference no. PCA/PA/778) were purchased from Tulsi Amrit Pvt. Ltd (Indore, India) and Natural Remedies Private Limited (Bangalore, India), respectively. Plant parts of *P. emblica* and *P. fraternus* were washed thoroughly with normal tap water followed by Milli-Q water and dried at room temperature (26–28 °C). All dried plants were crushed into powder using grinding machine (Decibel, Lab Willey Griender, and Model No. DB 5581-4, New Delhi, India) and stored in airtight container at room temperature until analysis.

2.3. Extraction

Each sample (5 g) was dipped with ethanol (15 mL) followed by 30 min sonication at 30 °C and kept for 48 h at the room temperature. The ethanol extracts were filtered by Whitman No. 1 filter paper and filtrate was concentrated under reduced pressure at 20–50 kPa at 40 °C using a Buchi rotary evaporator [22]. This procedure was applied three times with fresh solvent. All extracts were stored in the refrigerator at –20 °C until analysis. Each extract (approximately 1 mg) was weighed accurately and dissolved in methanol accordingly to prepare 1 mg/mL stock solution.

2.4. HPLC-ESI-QTOF-MS/MS conditions

Analyses were carried out using an Agilent 1200 HPLC system interfaced with Agilent 6520 hybrid quadrupole time of flight mass spectrometer (Agilent technologies, USA). 1200 HPLC system was equipped with quaternary pump (G1311A), online vacuum degasser (G1322A), autosampler (G1329A), column compartment (G1316C) and diode-array detector (G1315D).

2.4.1. Chromatographic conditions

Chromatographic separations were performed using a Thermo Betasil C_8 column (250 mm×4.5 mm, 5 µm) operated at 25 °C employing a gradient elution using 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) as mobile phase at a flow rate of 0.4 mL/min. The elution consisted of a gradient of 35%–90%, 0–7 min, 90%–90%, 7–25 min, 90%–35%, 25–35 min and initial condition was maintained for 5 min. The sample injection volume was 1 µL.

2.4.2. Mass spectrometric condition

Mass spectrometer was operated in negative electrospray ionization mode and spectra were recorded by scanning the mass range from *m/z* 50 to 1500 in both MS and MS/MS modes. Nitrogen was used as drying, nebulising and collision gas. Drying gas flow rate was 12 L/min. The heated capillary temperature was set at 350 °C and nebulizer pressure at 45 psi. The source parameters such as capillary voltage (VCap), fragmentor, skimmer and octapole voltages were set at 3500 V, 175 V, 65 V and 750 V, respectively. For the MS/MS analysis, collision energies were set at 15, 20, 25, 30, 35 and 40 eV. The accurate mass data of the molecular ions were processed through the Mass Hunter Workstation (version B 04.00) software.

3. Results

3.1. LC–MS/MS analysis of flavonoids

MS/MS spectra of selected flavonol-*O*-glucosides were analyzed at different collision energies (5–50 eV) shown in Fig. 2 and Fig. S1. Rutin (16), quercetin-3,4-di-*O*-glucoside (26) and kaempferol-3-*O*-rutinoside (28) were selected as templates. Compounds 16, 26 and 28 showed abundant $[Y]^-$ ions at collision energies of 35, 20 and 30 eV, respectively, in MS/MS analysis. The abundance of $[Y_1]^-$ ion was decreased with increased abundance of $[Y\text{-}H]^-$ ions at high collision energies (Fig. S1). Thus, flavonol-*O*-glucosides also showed abundant $[Y\text{-}H]^-$ product ion at high collision energy [40–43].

3.2. Screening of bioactive compounds

To achieve satisfactory separation, the ethanolic extracts were analyzed using gradient mobile phase consisting of 0.1% formic acid in methanol and aqueous formic acid (0.1% formic acid) after optimization. Different column types, column temperature, mobile phase, elution conditions, flow rates and MS conditions were also optimized. Base peak chromatograms (BPCs) of *P. emblica* (A, B and C), *P. amarus* (D), *P. fraternus* (E, F and G), and *P. niruri* (H) in negative ionization mode are shown in Fig. 3. Retention time (RT), observed $[M\text{-}H]^-$, molecular formula, error (Δppm), major fragment ions and their relative abundance and distribution along with assignment are presented in Tables 1–3.

Eleven compounds, namely, 3 (quercetin 3-*O*-hexoside), 16 (rutin), 18 (quercetin 3-isorhamninoside), 19 (quercetin derivative), 20 (quercetin derivative), 21 (quercetin-di-*O*-hexoside), 24 (quercetin 3-sambubioside), 26 (qauercetin-3,4-di-*O*-glucoside), 30 (quercetin-*O*-hexoside), 32 (quercetin 3-arabinoside) and 33 (quercetin 3-*O*-glucuronide), were identified as quercetin derivatives. All these compounds (3, 16, 18, 19, 20, 21, 24, 26, 30, 32 and 33) showed characteristic fragment ion at *m/z* 301 $[Y]^-$ due to elimination of $C_6H_{10}O_4$, $C_{12}H_{20}O_9$, $C_{18}H_{30}O_{13}$, $C_{12}H_{18}O_{10}$, $C_{12}H_{20}O_8$, $C_{12}H_{20}O_{10}$, $C_{11}H_{18}O_9$, $C_{12}H_{20}O_{10}$, $C_6H_{10}O_5$ $C_5H_8O_4$ and $C_6H_8O_6$, respectively. Further loss of H radical from $[Y]^-$ ion generated radical ion $[Y\text{-}H]^{-\bullet}$ at *m/z* 300 [44]. Similarly, all these compounds and 45 (quercetin) produced fragment ions at *m/z* 271 and 255 due to loss of $[Y\text{-}CHO]^-$ and $[CO+H_2O]^-$, respectively. Identification of compounds 16, 26 and 45 was also confirmed by comparison of RT and MS/MS spectra with the authentic standards. Compounds 21 and 26 were isomers which showed the same MS/MS fragment ions with different relative abundance at 13.04 and 13.42 min, respectively. All compounds also showed Retro Diels Alder (RDA) fragment ion at *m/z* 151 due to B-ring cleavage (Table 1).

Seven compounds, namely, 25 (robinin), 28 (kaempferol-3-*O*-rutinoside), 29 (kaempferol- hexoside), 38 (kaempferol-3-*O*- hexoside), 40 (kaempferol derivatives), 41 (kaempferol 3-*O*-glucuronide) and 43 (kaempferol-*O*-hexoside), were identified as kaempferol derivatives. The characteristic fragment ion at *m/z* 285 $[Y]^-$ was observed in all the compounds (25, 28, 29, 38, 40, 41 and 43) due to loss of $C_{18}H_{31}O_{13}$, $C_6H_{10}O_5$, $C_{12}H_{20}O_9$, $C_6H_{10}O_5$, $C_5H_8O_4$, $C_6H_8O_6$ and $C_6H_{10}O_4$, respectively. Fragment ion $[Y\text{-}H]^-$ was observed as a radical anion at *m/z* 284 due to loss of H radical. All these compounds and 48 (kaempferol) produced fragment ions at *m/z* 255 and 227 due to loss of CHO and 2CHO. Compounds 28 and 48 were also confirmed with authentic standards (Table 2).

Compounds 1, 2, 4, 37 and 44 were identified as quinic acid, caffeic acid, gallic acid, coumaric acid and protocatechuic acid by comparison of RT and MS/MS with their standards. MS/MS spectra of compounds 2, 4, 37 and 44 showed fragment ions at *m/z* 135, 125, 119 and 109, respectively due to loss of CO_2. Compound 5 was identified as vanillic acid which showed fragment ions at *m/z* 151 and 123 due to loss of CH_3 and CO_2, respectively. Fragment ions at *m/z* 151 and 123 produced common fragment ion at *m/z* 107 due to losses of HCO_2 and CH_4, respectively. Compound 6 was identified as gentisic acid-*O*-hexoside which showed fragment ion at *m/z* 152 due to loss of hexoside.

Compounds 9 (methyl gallate) and 23 (ethyl gallate) were identified as gallates of gallic acid which gave characteristic fragment ion at *m/z* 169 due to loss of CH_3 and C_2H_5, respectively. MS/MS spectra of both compounds showed fragment ion at *m/z* 125 as base peak. Compound 19 was identified as brevifolin and other compounds 4 (brevifolincarboxylic acid), 14 (methyl brevifolincarboxylate), 26 (ethyl brevifolincarboxylate), and 28 (propyl-*O*-methyl brevifolin) were its derivatives. Compound 4 showed fragment ion at *m/z* 247 due to loss of CO_2 whereas fragment ions at *m/z* 219, 191 and 175 were observed due to successive losses of CO. Fragment ion at *m/z* 273 was observed in compounds 14 and 26 due to loss of CH_3OH and C_2H_5OH, respectively whereas other fragment ions were formed due to consecutive loss of CO. Compound 19 also showed major fragment ions at *m/z* 219 and 191 due to consecutive loss of CO. Similarly, compound 28 showed fragment ions at *m/z* 247 and 245 due to loss of C_3H_7 and CO_2, respectively. Compounds 7, 9 and 14 were identified as catechin and epicatechin catechin 3-gallate, respectively. Compound 7 and 9 were isomers and showed the same fragment ions with different relative abundance. They were also confirmed by comparison with their standards.

Seven compounds, namely, 10 (ellagic acid-*O*-dihexoside), 11 (ellagic acid-*O*-hexoside), 12 (ellagic acid-*O*-glucuronide), 23 (ellagic acid-*O*-arabinoside), 42 (methy-*O*-ellagic acid), 47 (dimethyl-*O*-ellagic acid) and 49 (trimethyl-*O*-ellagic acid), were identified as ellagic acid derivatives. Compounds 10, 11, 12 and 23 showed characteristic fragment ion at *m/z* 300 due to loss of $C_{12}H_{20}O_{10}$, $C_6H_{10}O_5$, $C_6H_8O_6$ and $C_5H_8O_4$, respectively. Similarly, compounds 42, 47 and 49 showed fragment ions at *m/z* 299, 314 and 328, respectively, due to loss of CH_3. Compound 35 showed fragment ions at *m/z* 283 and 245 due to loss of H_2O and 2CO. Compounds 15, 35, 39, 42, 46, 50 and 51 were identified as chrysin, ellagic acid, eriodictyol, methy-*O*-ellagic acid, luteolin, betulinic acid and oleanolic acid, respectively and confirmed

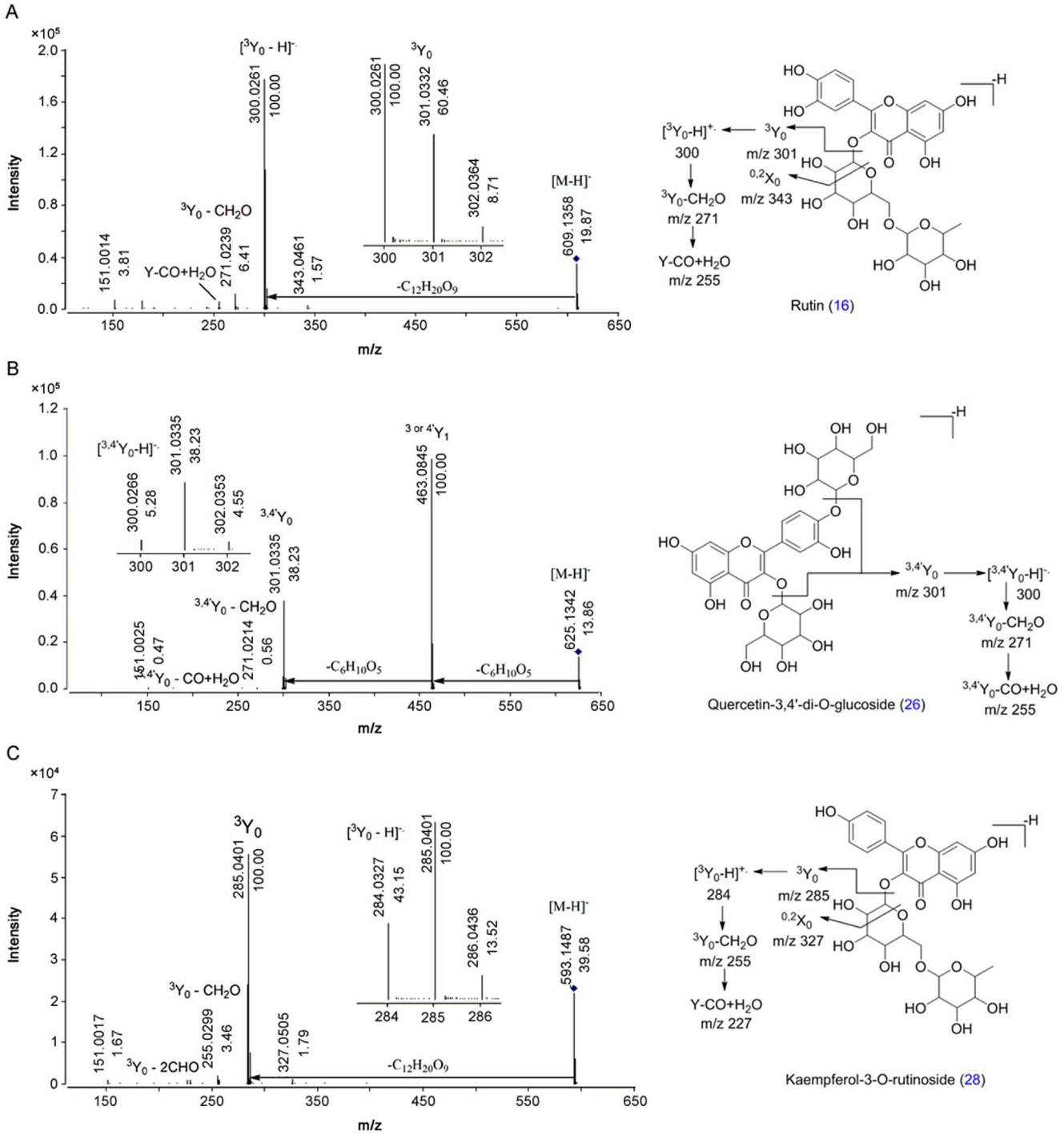

Fig. 2. MS/MS spectra of standards (A) rutin (16), (B) quercetin-3,4-di-*O*-glucoside (26) and (C) kaempferol-3-*O*-rutinoside (28) at collision energy 35, 20 and 30 eV, respectively.

by comparison of RT and MS/MS spectra with their standards (Table 3).

4. Discussion

Most of the qualitative and quantitative analyses of phenolics in *Phyllanthus* species are reported by HPLC or HPTLC based on their RT and UV data [17–19]. Identification and distribution of 15 compounds are reported in *P. amarus*, *P. stipulatus*, *P. niruri* and *P. tenellus* in 60 min based on unit mass resolution only [29]. Yang et al. [30] have also identified hydrolysable tannins and other phenolic compounds in 65 min from *P. emblica* fruit using HPLC-DAD-ESI(−)-QTOF-MS/MS [30]. Recently, fingerprinting and identification in *P. amarus* and *P. niruri* using LC–MS/MS analysis have been reported by some authors independently [31–33]. In the our previous report, 11 compounds (gallic acid, protocatechuic acid, caffeic acid, quercetin, ellagicacid, rutin, keamferol-3-*O*-rutinoside, luteolin, kaempferol, quinic acid and ursolic acid) were unambiguously identified and characterized whereas the other 41 compounds were tentatively identified and characterized. Only five most abundant compounds were quantified in ethanolic extracts of *P. amarus* samples collected from three different locations [35].

HPLC-ESI-QTOF-MS/MS facilitates the identification and characterization of known and unknown compounds on the basis of their molecular formula, exact mass measurements and MS/MS fragmentations [44,45]. It also differentiates isobaric compounds by exact masses

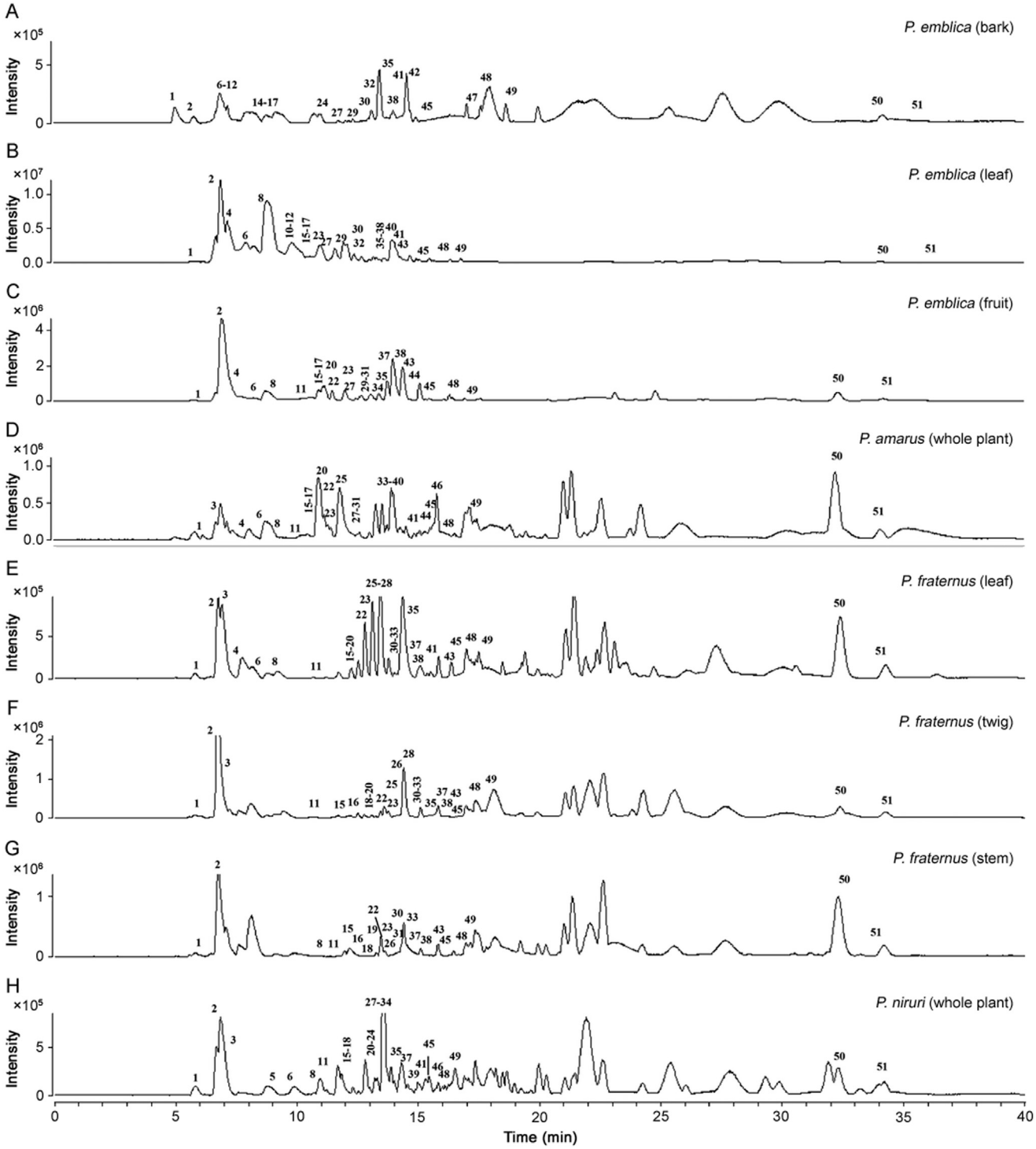

Fig. 3. Base peak chromatograms of (A) *P. emblica* (bark), (B) *P. emblica* (leaf), (C) *P. emblica* (fruit), (D) *P. amarus,* (E) *P. fraternus* (leaf), (F) *P. fraternus* (leaf), (G) *P. fraternus* (leaf), and (H) *P. niruri*.

with different elemental compositions. In addition, HPLC-ESI-QTOF-MS provides separation and targeted fragmentation of any particular ion of interest, which may contribute to structural elucidation and isomer distinction [44–46]. Analysis of phenolics is reported in positive and negative ionization modes [41,42]. But negative ionization mode is found more sensitive for the analysis of these compounds [35,40–43]. In the present work, we have selected four *Phyllanthus* species plants or parts, namely, *P. emblica, P. fraternus, P. amarus* and *P. niruri* which are commonly used as medicine. Therefore, the comparative fingerprints of *P. emblica, P. fraternus, P. amarus* and *P. niruri* were generated using HPLC-ESI-QTOF-MS/MS in negative ionization mode. Twenty-one compounds were unambiguously identified and characterized by comparison (RT and MS/MS spectra) with authentic standards whereas 30 compounds were tentatively identified and characterized with the help of templates (reference compounds). Exact mass measurements and characteristic diagnostic fragment ions were used to identify the compounds which are more accurate and authentic than earlier reported methods. This method was initially developed on *P. amarus* extract and applied on other selected plants to test its suitability. Results proved the applicability of the developed method

Table 1
Chromatographic and spectrometric data of quercetin and its derivatives identified compounds in ethanolic extracts of *Phyllanthus* species.

S. No.	Retention time (min)	Observed. $[M-H]^-$	Error (ppm)	Molecular formula	Fragment ions (Relative intensity %)				Compounds	Distribution							
					$[Y]^-$	$[Y-H]^{-\cdot}$	$[Y-CH_2O]^-$	$[Y-CO+H_2O]^-$		PEB	PEF	PEL	PA	PFL	PFT	PFS	PN
3	7.87	447.0947	−3.21	$C_{21}H_{20}O_{11}$	301.0323 (93)	300.0283 (100)	271.0248 (13)	255.0283 (28)	Quercetin 3-*O*-hexosde	−	−	−	+	+	+	+	+
16	12.26	609.1463	0.03	$C_{27}H_{30}O_{16}$	301.0342 (42)	300.0277 (100)	271.0251 (8)	255.0312 (5)	Rutin†	+	+	+	+	+	+	+	+
18	12.52	755.2039	1.02	$C_{33}H_{40}O_{20}$	301.0294 (94)	300.0287 (100)	271.0235 (11)	255.0367 (3)	Quercetin 3-isorhamninoside (FT)	−	−	−	−	+	+	+	+
19	12.75	623.1252	0.25	$C_{27}H_{28}O_{17}$	301.0328 (68)	300.0277 (100)	271.0222 (19)	255.0330 (8)	Quercetin derivative	−	−	−	−	+	+	+	−
20	13.00	593.1514	0.27	$C_{27}H_{30}O_{15}$	301.0324 (84)	300.0356 (100)	271.0345 (6)	255.0377 (5)	Quercetin derivative	−	−	+	+	+	+	−	+
21	13.04	625.1412	0.02	$C_{27}H_{30}O_{17}$	301.0344 (62)	300.0282 (100)	271.0228 (7)	255.0310 (3)	Quercetin-di-*O*-hexoside (isomer)	−	−	−	−	−	−	−	+
24	13.12	595.1282	3.87	$C_{26}H_{28}O_{16}$	301.0318 (61)	300.0251 (100)	271.0228 (7)	255.0429 (2)	Quercetin 3-sambubioside	−	−	−	−	−	−	−	+
26	13.42	625.1413	0.03	$C_{27}H_{30}O_{17}$	301.0328 (100)	300.0274 (54)	271.0245 (5)	255.0367 (2)	Quercetin-3,4-di-*O*-glucoside†	−	−	−	−	+	+	+	−
30	13.79	463.0944	1.02	$C_{21}H_{20}O_{12}$	301.0327 (63)	300.0257 (100)	271.0236 (10)	255.0271 (8)	Quercetin-*O*-hexoside	+	+	+	+	+	+	+	+
32	13.91	433.0772	0.65	$C_{20}H_{18}O_{11}$	301.0290 (49)	300.0286 (100)	271.0300 (15)	255.0231 (7)	Quercetin 3-arabinoside	+	+	−	−	+	+	−	+
33	13.96	477.0662	2.63	$C_{21}H_{18}O_{13}$	301.0336 (100)	300.0283 (100)	271.0248 (13)	255.0271 (1)	Quercetin 3-*O*-glucuronide	−	−	−	+	+	+	+	+
45	15.35	301.0358	0.03	$C_{15}H_{10}O_7$	–	–	271.0413 (1)	255.0271 (2)	Quercetin†	+	+	+	+	+	+	+	+

PEB: *P. emblica* bark; PEF: *P. emblica* fruit; PEL: *P. emblica* leaf; PA: *P. amarus*; PFL: *P. fraternus* leaf; PFT: *P. fraternus* twig; PFS: *P. fraternus* stem; PN: *P. niruri*

Table 2
Chromatographic and spectrometric data of kaempferol and its derivatives identified compounds in ethanolic extracts of *Phyllanthus* species.

S. No.	Retention time (min)	Observed. $[M-H]^-$	Error (ppm)	Molecular formula	Fragment ions (Relative intensity %)				Compounds	Distribution							
					$[Y]^-$	$[Y-H]^{-\cdot}$	$[Y-CH_2O]^-$	$[Y-2CHO]^-$		PEB	PEF	PEL	PA	PFL	PFT	PFS	PN
25	13.13	739.2092	0.20	$C_{33}H_{40}O_{19}$	285.0385 (65)	284.0319 (100)	255.0315 (35)	227.0332 (10)	Robinin	−	−	−	+	+	+	−	−
28	13.53	593.1551	−0.65	$C_{27}H_{30}O_{15}$	285.0303 (100)	284.0331 (78)	255.0299 (12)	227.0327 (3)	Kaempferol-3-*O*-rutinoside†	−	−	−	+	+	+	−	+
29	13.64	609.1462	0.05	$C_{27}H_{30}O_{16}$	285.0348 (100)	284.0304 (15)	255.0427 (25)	227.0542 (5)	Kaempferol-diglucoside	+	+	+	+	−	−	−	+
38	14.36	447.0930	0.65	$C_{21}H_{20}O_{11}$	285.0368 (43)	284.0315 (100)	255.0276 (23)	227.0332 (18)	Kaempferol 3-*O*-hexoside		+	+	+	+	+	+	
40	14.70	417.0828	0.06	$C_{20}H_{18}O_{10}$	285.0348 (30)	284.0304 (100)	255.0275 (36)	227.0329 (17)	Kaempferol derivatives	−	+	−	−	−	−	−	−
41	14.84	461.0722	−0.25	$C_{21}H_{18}O_{12}$	285.0390 (100)	284.0323 (14)	255.0453 (3)	227.0590 (5)	Kaempferol 3-glucuronide	+	+	−	+	+	−	−	+
43	15.14	431.0983	0.18	$C_{21}H_{20}O_{10}$	285.0437 (61)	284.0370 (100)	255.0299 (42)	227.0327 (19)	Kaempferol 3-hexoside	−	+	+	−	+	+	+	−
48	16.31	285.0477	0.54	$C_{15}H_{10}O_6$	–	–	255.0219 (32)	227.0356 (45)	Kaempferol†	+	+	+	+	+	+	+	+

PEB: *P. emblica* bark; PEF: *P. emblica* fruit; PEL: *P. emblica* leaf; PA: *P. amarus*; PFL: *P. fraternus* leaf; PFT: *P. fraternus* twig; PFS: *P. fraternus* stem; PN: *P. niruri*

Table 3
Chromatographic and spectrometric data of other identified classes of compounds in ethanolic extract of *Phyllanthus* species.

S. No.	Retention time (min)	Observed. $[M-H]^-$	Error (ppm)	Molecular formula	Fragment ions (Relative intensity %)	Compounds	Distribution							
							PEB	PEF	PEL	PA	PFL	PFT	PFS	PN
1	6.01	191.0565	0.26	$C_7H_{12}O_6$	127.0428, 109.0315, 93.0369, 85.0318 (100), 81.0366, 59.0165, 43.0214	Quinic acid†	+	+	+	+	+	+	+	+
2	7.27	179.0380	0.12	$C_9H_8O_4$	135.0444 (100)	Caffeic acid†	+	+	+		+	−	−	+
4	9.16	169.0130	1.02	$C_7H_6O_5$	125.0232 (100)	Gallic acid†	−	+	+	+	+	+	−	−
5	10.28	167.0370	0.41	$C_8H_8O_4$	151.0003 (9), 123.0441 (67), 107.0105 (9), 95.0472 (11), 83.012 (67), 81.0337 (100), 63.0250 (6), 57.0366 (13)	Vanillic acid†	−	−	−	−	−	−	−	+
6	10.53	315.0713	3.19	$C_{13}H_{16}O_9$	152.0093 (100), 108.0215 (55)	Gentisic acid-*O*-hexoside	+	+	+	+	+	−	−	+
7	10.55	289.0718		$C_{15}H_{14}O_6$	247.0240 (100), 205.0494 (9), 151.0391 (6), 125.0231 (10)	(+)-catechin†	+	+	−	−	−	−	−	−
8	11.68	291.0148	−0.72	$C_{13}H_8O_8$	247.0228 (100), 219.0282 (11), 191.0336 (23), 175.0336 (6)	Brevifolincarboxylic acid	+	+	+	+	+	−	−	+
9	11.74	289.0720	0.54	$C_{15}H_{14}O_6$	247.0229 (72), 245.0812 (32), 221.0795 (22), 203.0695 (62), 161.0584 (28), 151.0375 (49), 137.0221 (35), 125.0226 (67), 123.0434 (69), 109.0281 (100)	Epicatechin†	+	−	−	−	−	−	−	−
10	11.85	625.1046	0.23	$C_{26}H_{26}O_{18}$	300.9990 (100)	Ellagic acid-*O*-dihexoside	+	+	−	−	−	−	−	−
11	11.99	463.0518	0.12	$C_{20}H_{16}O_{13}$	300.9979 (100), 299.9909 (51), 243.9948 (3)	Ellagic acid-*O*-hexoside	+	+	+	+	+	+	+	+
12	12.15	477.0310	1.02	$C_{10}H_{10}O_4$	300.9967 (86)	Ellagic acid-*O*-glucuronide	+	+	−	−	−	−	−	−
13	12.18	194.0578	−0.12	$C_{10}H_{10}O_4$	178.0266 (45), 134.0362 (100)	Ferulic acid†	−	−	−	−	−	−	−	+
14	12.22	441.0829	0.12	$C_{22}H1_8O_{10}$	289.0686 (34), 245.0787 (16), 169.021 (100), 125.0214 (23)	Catechin 3-gallate	+	−	−	−	−	−	−	−
15	12.23	253.0506	0.23	$C_{15}H_{10}O_4$	152.0124 (100),	Chrysin†	+	+	+	+	+	+	+	+
17	12.29	183.0299	−0.02	$C_8H_8O_5$	169.016 (14), 124.0131 (100)	Methyl gallate	+	+	+	+	+	−	−	+
22	13.05	305.0305	0.81	$C_{14}H_{10}O_8$	273.0022 (3), 245.0091 (33), 217.0137 (100), 201.0193 (15), 189.0189 (58), 161.0243 (82), 145.0286 (44), 133.0292 (63)	Methyl brevifolincarboxylate	−	−	+	+	+	+	+	+
23	13.11	433.0411	0.42	$C_{19}H_{14}O_{12}$	300.9964 (100), 299.9934 (81), 271.9887 (2), 243.9948 (3)	Ellagic acid-*O*-arabinoside	+	+	+	+	+	+	+	+
27	13.44	247.0248	2.23	$C_{12}H_8O_6$	219.0309 (38), 191.0352 (100), 173.0249 (52), 145.0297 (79)	Brevifolin	+	+	+	+	+			+
31	13.83	197.0444	5.93	$C_9H_{10}O_5$	169.0131 (16), 124.0156 (100)	Ethyl gallate	−	−	+	+	+	+	+	+
34	13.98	319.0459	2.29	$C_{15}H_{12}O_8$	273.0040 (62), 245.0099 (100), 229.0154 (10), 217.0142 (100), 201.0201 (11), 189.0194 (14)	Ethyl brevifolincarboxylate	−	−	+	+	−	−	−	−
35	14.07	300.999	0.33	$C_{14}H_6O_8$	283.9975 (66), 245.0085 (36), 229.0135 (45), 200.0103 (58), 185.0242 (39), 173.0232 (60), 145.0299 (100)	Ellagic acid†	+	+	+	+	+	+	−	+
36	14.12	289.0718	1.23	$C_{15}H_{14}O_6$	247.0242 (100), 245.0817 (8), 221.0851 (52), 203.0660 (67), 151.0374 (68)	Propyl-*O*-methyl Brevifolin	−	+		+	−	−	−	−
37	14.34	163.0411	1.02	$C_9H_8O_3$	119.0414 (100)	Coumaric acid†	+	+	+	+	+	+	+	+
39	14.56	287.0586	1.23	$C_{15}H_{12}O_6$	151.0029 (100), 135.0446 (86), 125.0240 (4), 107.0138 (19)	Eriodictyol†	−	−	−	+	−	−	−	+
42	15.06	315.0149	0.93	$C_{15}H_8O_8$	299.9888 (100)	Methy-*O*-ellagic acid†	+	−	−	−	−	−	−	−
44	15.19	153.0196	1.39	$C_7H_6O_4$	109.0284 (100)	Protocatechuic acid†	−	−	+	+	−	−	−	−
47	16.08	329.0304	−0.40	$C_{16}H_{10}O_8$	314.0060 (100), 298.9824 (24), 270.9910 (8)	Dimethyl-*O*-ellagic acid	+	−	−	−	−	−	−	−
49	18.14	343.0416	0.23	$C_{17}H_{12}O_8$	328.0259 (17),312.9999 (100), 297.9739 (64), 285.0023 (18), 269.9803 (19)	Trimethyl-*O*-ellagic acid	+	+	+	+	+	+	+	+
46	15.91	285.0405	0.12	$C_{15}H_{10}O_6$	151.0035 (31), 133.0289 (100), 121.0290 (4), 107.0149 (15)	Luteolin†	−	−		+	−	−	−	+
50	33.18	455.3535	0.21	$C_{30}H_{48}O_3$		Betulinic acid†	+	+	+	+	+	+	+	+
51	34.03	455.3537	0.34	$C_{30}H_{48}O_3$	307.3314 (7)	Oleanolic acid†	+	+	+	+	+	+	+	+

PEB: *P. emblica* bark; PEF: *P. emblica* fruit; PEL: *P. emblica* leaf; PA: *P. amarus*; PFL: *P. fraternus* leaf; PFT: *P. fraternus* twig; PFS: *P. fraternus* stem; PN: *P. niruri*

† matched with reference compounds

on various plants/parts of *Phyllanthus* species. Distribution of all the compounds is also reported according to the plant parts.

5. Conclusion

Optimization of suitable collision energies and identification of diagnostic fragment ions of rutin, quercetin-3,4-di-*O*-glucoside and kaempferol-3-*O*-rutinoside were successfully completed. HPLC-ESI-QTOF-MS/MS method was developed for the identification, characterization and distribution of phenolics on the basis of identified diagnostic fragment ions of flavonoides and reported diagnostic fragment ions of phenolic acids and other compounds in 35 min run time in the crude extracts of *Phyllanthus* species plants/parts. Total 51 compounds including 21 were unambiguously identified and characterized on comparison with their standards whereas remaining 30 were tentatively identified and characterized. Most of these compounds are reported for the first time in *P. fraternus* and *P. niruri*.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2017.01.005.

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