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HPLC-ESI-MS/MS Profiling of Phenolic Acids, Flavonoids And Sesquiterpene Lactones from *Xanthium spinosum*

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Abstract*. Bathurst burr (Xanthium spinosum L.) is used worldwide in traditional medicine to treat a diverse range of health problems including urinary problems associated with various prostate diseases. The aim of this study was to complete the identification and structural characterization of the chemical constituents from the aerial part of X. spinosum by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. From the unequivocally detected and characterized compounds of aqueous-methanol extract, protocatechuic acid, 4-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid were found for the first time in X. spinosum. Besides these compounds, 25 phenolics (including 7 hydroxybenzoic derivatives, 13 hydroxycinnamic derivatives, one benzyl alcohol-hexose-pentose, and 4 flavonoids), 6 sesquiterpenes and 3 diterpenes were tentatively identified.*

Keywords*: Xanthium spinosum, protocatechuic acid; 4-O-caffeoylquinic acid; 3,5-di-O-caffeoylquinic acid; HPLC-ESI-MS/MS*

1.Introduction

Bathurst burr (*Xanthium spinosum* L.), from the family Asteraceae is an annual herb originated from South America. The herb is used traditionally in Romania for urinary problems and various prostate diseases [1]. The pharmacological profile of *X. spinosum* includes the anti-inflammatory activity attributed to its flavonoids. The sesquiterpene lactone xanthatin from the leaves has antibacterial and antifungal properties [2, 3]. Xanthatin extracted from the aerial parts of the plant shows inhibitory activity against a wide variety of viruses. Also, it was revealed that xanthatin has a strong anti-angiogenesis capacity *in vitro* [4]. The infusion and tincture obtained from *X. spinosum* aerial parts are efficient in the treatment of rats with induced benign prostate hypertrophia [5, 6].

The revealed chemical constituents of *X. spinosum* were classified into three main groups of active principles [7]: phenolics (caffeic acid, chlorogenic acid, quercetin, pendulin, jacein, centaurein, and patuletin-3-*O*-glycoside), sesquiterpenes (2-acetoxi-4-*O*- hydroxideacetylxanthanol, 1α,5α-epoxy-1,5-dihydroxanthatin, 1β,5β-epoxy-1,5-dihydroxanthatin, desacetyl xanthiuminol, 2-hydroxi-4-*O*-acetoxi-deacetylxanthanol, 2-hydroxi-4-oxo-deacetylxanthanol, 2-*O*-acetyl-4-oxo-desacetylxanthanol, 2-oxo-4-*O*-acetyldesacetylxanthanol, xanthatin, xanthinin, 2-desacetyl-8-epi-xanthumanol-4-*O*-β-D-galacto-pyranoside, xanthumin, deacetilxanthumin, stizolicin and solstitialin), and diterpenes

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(3', 4'-didesulphated-carboxyatractyloside, 3', 4'-didesulphated-atractyloside, 4'-desulphated- carboxy-atractyloside, 4'-desulphated-atractyloside, atractyloside, 2β-*O*-β-D-glucopyranosyl- 15α-hydroxy-kaur-16-en-18,19-dicarboxylic acid).

The presence of toxic kaurene glycosides prevented the researcher from further exploration of therapeutic potential of the genus *Xanthium*. However, in a recent study the anti-diabetic actions of individual compounds isolated from *X. strumarium* were screened. Caffeoylquinic acid derivatives and phenolic constituents obtained from the plant had inhibitory effect on aldose reductase, α-glucosidase, protein tyrosine phosphatase 1β, and advanced glycation end products [8]. In the light of these results, the aim of this study was to complete the identification of the chemical constituents from the aerial parts of *X. spinosum* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS).

2.Materials and methods

Plant material

The flowering aerial parts of *Xanthium spinosum* L. were collected at the end of October 2014 from the Botanical Garden belonging to the University of Medicine, Pharmacy, Sciences and Technology "George Emil Palade" from Târgu Mureș, Romania (46°33'16.91"N, 24°35'0.51"E). The plant was identified and the voucher specimen (GB/H/0162/2014) is deposited at the Faculty of Pharmacy, Department of Pharmaceutical Botany.

Chemicals and solvents

The chemical reference substances: 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid (cynarin), 3,5-dicaffeoylquinic acid, caffeic acid, and protocatechuic acid were purchased from Sigma-Aldrich (St. Louis, USA), while xanthatin was bought from ChemFaces (Wuhan, China). These standards were chosen, after the phytochemical exploring of the *Xanthium* genus. Acetonitrile and methanol were of HPLC super gradient grade (Sigma-Aldrich). Water was purified with Millipore (Billerica, MA, USA) Milli-Q equipment. All other chemicals were of analytical reagent grade.

Extraction

The powdered herb of *X. spinosum* (2 g) was extracted twice with 20 mL of 8:2 (*v/v*) methanol:water by sonication (Braun Labsonic U, Melsungen, Germany) for 2 × 5 min. The extracts were separated from the plant powder by centrifugation at 6000 rpm (2500 g) for 10 min. The two methanolic fractions were combined and evaporated to dryness under reduced pressure (Rotavapor, R-200, Büchi, Flawil, Switzerland) below 60°C before purification by solid-phase extraction (SPE).

SPE was performed on Supelclean LC-18 micro-columns (500 mg, 3 mL), supplied by Supelco (Bellefonte, PA, USA) using a 12-port vacuum manifold processor (LiChrolut extraction unit; Merck). Samples were dissolved in 80% (*v/v*) aqueous methanol and were loaded on to the SPE micro columns previously activated by 5 mL methanol, 5 mL water and 2.5 mL methanol. The collected eluate was completed by a further portion of the extract obtained by loading 2.5 mL of 80% methanol on to the SPE column. Finally, the samples obtained were evaporated to dryness and were dissolved in 2 mL of 80% methanol.

HPLC-DAD-ESI-MS/MS analysis

For chromatographic separation and mass spectral analysis, an Agilent 1100 HPLC system (degasser, binary gradient pump, autosampler, column thermostat and diode array detector) was used coupled with an Agilent 6410 Triple Quad LC/MS system equipped with ESI ion source (Agilent Technologies, Palo Alto, CA, USA).

HPLC separations were achieved on a Zorbax SB-C18, Solvent Saver Plus (3.5 μm, 80 Å) reversed-phase column (150 mm x 3.0 mm i.d.; Agilent Technologies, Santa Clara, CA, USA). Mobile phase consisted of 0.3% (*v/v*) aqueous formic acid (A) and methanol supplemented with 0.1% (*v/v*) formic acid (B). The following gradient program was applied: 0.00 min, 10% B; 15.00 min, 67.5% B; 24.00 min, 67.5% B; 25.00 min, 100% B; 29.00 min; 100% B; 32.00 min, 10% B. The solvent flow rate was 0.4 mL/min, and column temperature was set at 25 °C. The injection volume was 4 μL.

Electrospray conditions were as follows: drying gas (N_2) temperature, 350 °C; flow rate, 9 L/min; nebulizer pressure, 45 psi (N_2); fragmentor voltage, 100V; capillary voltage, 3500V. High purity nitrogen was used as collision gas, and collision energy was changed between 5 and 60 eV according to differences in molecule structures. Full scan mass spectra were recorded in positive and negative ion mode over an *m/z* range of 50-1000.

3. Results and discussions

The HPLC-DAD-ESI-MS/MS analysis of the aqueous-methanolic extract of the aerial part of *Xanthium spinosum* revealed the presence of the following unequivocally detected constituents: protocatechuic acid (3), 4-*O*-caffeoylquinic acid (13), caffeic acid (15), 3,5-di-*O*-caffeoylquinic acid (22), and xanthatin (35) (Table 1). Three of the five unequivocally detected compounds are mentioned for the first time in *X. spinosum*: protocatechuic acid, 4-*O*-caffeoylquinic acid, and 3,5-di-*O*-caffeoylquinic acid. Protocatechuic acid and 3,5-di-*O*-caffeoylquinic acid were also detected in the aerial part of *X. strumarium*, with a yield of 25.3 mg and 5.8 mg, respectively [8]. According to their study, 3,5-di-*O*-caffeoylquinic acid has an inhibitory effect on α-glucosidase (antihyperglycemic activity), while protocatechuic acid presents inhibitory effect on $ABTS^+$ radical scavenging activity. Caffeoylquinic acids derivatives are the major active principles in the fruits of *X. strumarium*, and are known for their antioxidant, antibacterial and anti-inflammatory effects [26]. Therefore, further analyses are necessary to determine the content of the detected constituents, and to test their biological effects.

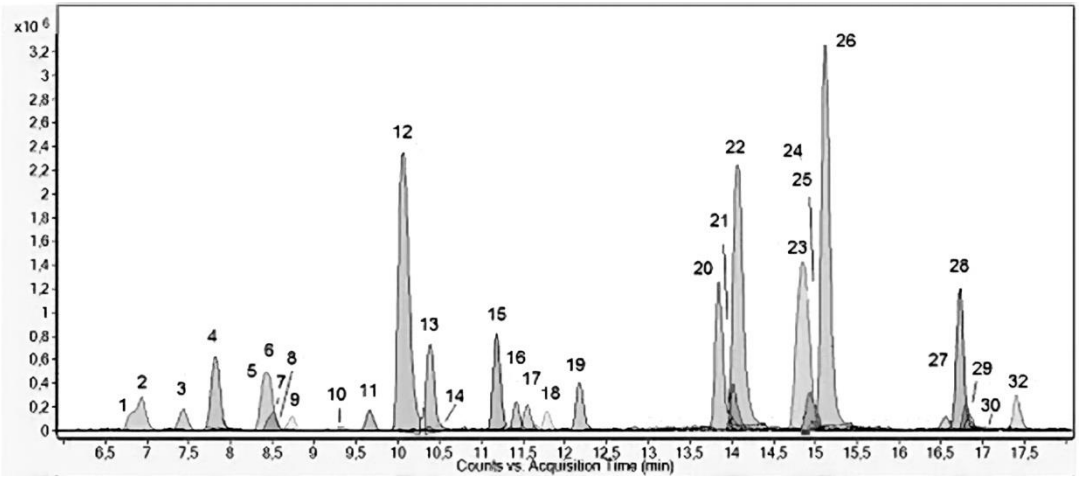

Figure 1. Overlaid extracted ion chromatogram (EIC) acquired in negative ion mode for the aqueous-methanolic extract of *Xanthium spinosum* aerial part. Peaks refer to Table 1

Table 1. Peak assignments of compounds detected in aqueous-methanolic extract of *Xanthium spinosum* aerial part, using fragmentation pattern of the precursor ions and their diagnostic product ions (negative or positive ion mode).

Peak	Identified compound	t_R (min)	$[M-H]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	Molecular formula	Fragment ions (*m/z*) (*RA%*)	Reference
	Hydroxybenzoic derivatives						
1	Protocatechuic acid-*O*-glucoside (3)	6.82	-	315	$C_{13}H_{16}O_9$	315 (30), 153 (37), 152 (100), 109 (21), 108 (38)	Kelebek et al., 2015 [9]
2	Protocatechuic acid-*O*-glucoside (4)	6.93	-	315	$C_{13}H_{16}O_9$	315 (24), 153 (34), 152 (100), 109 (13), 108 (31)	Kelebek et al., 2015
3	Protocatechuic acid	7.44	-	153	$C_7H_6O_4$	109 (100), 108 (32), 91 (5)	**Standard**
5	Protocatechuyl-glucose (3)	8.50	-	315	$C_{13}H_{16}O_9$	315 (5), 153 (100), 152 (29), 109 (57)	Kelebek et al., 2015
6	Protocatechuyl-glucose (4)	8.56	-	315	$C_{13}H_{16}O_9$	315 (27), 153 (100), 152 (14), 109 (76)	Kelebek et al., 2015
7	Dihydroxybenzoyl-hexose-pentose	8.58	-	447	$C_{18}H_{26}O_{14}$	163 (5), 152 (100), 109 (6), 108 (32)	Tahir et al., 2013 [10]
8	Dihydroxybenzoyl-hexose-pentose	8.62	-	447	$C_{18}H_{26}O_{14}$	163 (7), 152 (100), 109 (15), 108 (20)	Tahir et al., 2013
11	*p*-Hydroxybenzoyl-glucose	9.76	-	299	$C_{13}H_{16}O_8$	137 (100), 93 (26)	Allen et al., 2015 [11]
	Hydroxycinnamic derivatives						
4	3-*O*-Caffeoylquinic acid	7.82	-	353	$C_{16}H_{18}O_9$	191 (100), 179 (62), 135 (27)	Clifford et al., 2003 [12]
9	1-*O*-*p*-Coumaroylquinic acid	8.82	-	337	$C_{16}H_{18}O_8$	191 (100), 163 (7), 99 (6), 93 (6)	Clifford et al., 2007 [13]
10	3-*O*-*p*-Coumaroylquinic acid	9.43	-	337	$C_{16}H_{18}O_8$	191 (19), 163 (100), 119 (12)	Clifford et al. 2003
12	5-*O*-Caffeoylquinic acid	10.07	-	353	$C_{16}H_{18}O_9$	191 (100)	Clifford et al., 2003
13	4-*O*-Caffeoylquinic acid	10.38	-	353	$C_{16}H_{18}O_9$	191 (56), 179 (78), 173 (100), 135 (33)	**Standard**
14	5-*O*-*p*-Coumaroylquinic acid	10.59	-	337	$C_{16}H_{18}O_8$	191 (100)	Clifford et al., 2003
15	Caffeic acid	11.18	-	179	$C_9H_8O_4$	179 (17), 135 (100)	**Standard**
16	*cis*-5-*O*-Caffeoylquinic acid	11.41	-	353	$C_{16}H_{18}O_9$	191 (100)	Ncube et al., 2014 [14]
18	4-*O*-*p*-Coumaroylquinic acid *cis*-5-*O*-*p*-Coumaroylquinic acid	11.89	-	337	$C_{16}H_{18}O_8$	191 (100), 173 (52), 163 (13)	Clifford et al., 2003
19	5-*O*-Feruoylquinic acid	12.17	-	367	$C_{17}H_{20}O_9$	193 (10), 191 (100), 173 (17)	Clifford et al., 2003
20	3,4-di-*O*-Caffeoylquinic acid	13.84	-	515	$C_{25}H_{24}O_{12}$	515 (34), 353 (100), 335 (22), 191 (26), 179 (68), 173 (87), 161 (6), 135 (5)	Clifford et al., 2003
22	3,5-di-*O*-Caffeoylquinic acid	14.06	-	515	$C_{25}H_{24}O_{12}$	353 (91), 191 (100), 179 (60)	**Standard**
26	4,5-di-*O*-Caffeoylquinic acid	15.12	-	515	$C_{25}H_{24}O_{12}$	353 (100), 191 (14), 179 (45), 173 (63)	Clifford et al., 2003 Han et al., 2009 [15]
27	*cis*-4,5-di-*O*-Caffeoylquinic acid	16.56	-	515	$C_{25}H_{24}O_{12}$	353 (100), 191 (11), 179 (67), 173 (52)	Jaiswal et al., 2010 [16]
28	3,4,5-tri-*O*-Caffeoylquinic acid	16.80	-	677	$C_{34}H_{30}O_{15}$	677 (23), 515 (100), 353 (16)[a]; 335 (16), 191 (5), 179 (75), 173 (100), 161 (9)[b]	Gouveia and Castilho, 2012 [17]
	Benzyl alcohol derivatives						
17	Benzyl alcohol-hexose-pentose (formate adduct)	11.54	-	447	-	401 (18), 269 (100), 161 (79), 159 (7), 101 (15)	Spínola et al., 2014 [18]
	Flavonoids						
23	Quercetin-3-*O*-glucuronide	14.90	-	477	$C_{21}H_{18}O_{13}$	301 (94), 283 (13), 273 (17), 255 (18), 245 (14), 179 (58), 163 (23), 151 (100)	Hwang et al., 2016 [8]
24	Quercetin 3-*O*-(6-*O*-rhamnosyl- glucoside) (rutin)	14.99	-	609	$C_{27}H_{30}O_{16}$	301 (12), 300 (100), 271 (10), 151 (6)	Chen et al., 2015 [19]
25	Quercetin-3-*O*-glucoside (isoquercitrin)	15.04	-	463	$C_{21}H_{20}O_{12}$	300 (83), 271 (100), 255 (59), 179 (20), 151 (24)	Liao and Ku, 2012 [20]
	Sesquiterpenes						
33	xanthinosin derivative	17.82	249	-	-	231 (38), 213 (8), 207 (20), 203 (36), 189 (30), 185 (37), 175 (100), 161 (33), 159 (15), 157 (58), 145 (19), 133 (22)	Marco et al., 1993 [21]
34	isoxanthanol	17.88	309	-	$C_{17}H_{24}O_5$	291 (26), 273 (9), 263 (6), 249 (17), 231 (11), 213 (8), 205 (16), 203 (18), 189 (73), 185 (44), 171 (14), 159 (18), 143 (14)	Marco et al., 1993
35	xanthatin	18.01	247	-	$C_{15}H_{18}O_3$	229 (53), 211 (24), 205 (43), 201 (55), 187 (75), 183 (100), 159 (63), 131 (35)	**Standard**

36	xanthinosin derivative	18.19	249	-	-	231 (43), 213 (21), 203 (48), 189 (24), 187 (16), 185 (57), 175 (37), 161 (54), 159 (85), 147 (67), 145 (90), 133 (47), 131 (47)	Marco et al., 1993
37	8-epi-xanthatin	18.51	247	-	$C_{15}H_{18}O_3$	229 (73), 211 (24), 205 (28), 201 (68), 187 (53), 183 (71), 173 (100), 159 (90), 145 (41), 131 (64)	Chen et al., 2013 [22]
38	1,5-epoxy-1,5-dihydroxanthatin	22.45	263	-	$C_{15}H_{18}O_4$	235 (9), 217 (28), 199 (17), 189 (59), 175 (12), 161 (23), 147 (36), 133 (27), 123 (100)	Abdei-Mogib et al., 1991 [23]
	Diterpenes						
21	2-*O*-glucopyranosyl- carboxyatractyligenin	14.01	-	525	$C_{26}H_{38}O_{11}$	481 (100), 301 (7), 119 (13), 89 (9)	Lang et al., 2013 [24]
29	4'desulphated-carboxyatractyloside	16,82	-	689	$C_{21}H_{45}O_{13}S$	645 (83), 627 (100), 565 (16), 525 (8), 343 (10), 301 (13), 241 (5)	Carlier et al., 2014 [25]
32	3',4'-didesulphated- carboxyatractyloside	17,42	-	609	$C_{31}H_{46}O_{12}$	565 (100), 463 (41), 301 (28), 161 (8), 143 (6), 113 (15), 101 (7)	Carlier et al., 2014

Besides these compounds, 25 phenolics (including 7 hydroxybenzoic derivatives, 13 hydroxycinnamic derivatives, one benzyl alcohol-hexose pentose, and 4 flavonoids), 6 sesquiterpenes and 3 diterpenes were tentatively identified. The detected compounds with the main chromatographic and mass spectrometric data are listed in Table 1. Negative ion mode was used for phenolics and diterpenes (Figure 1), and positive ion mode for sesquiterpenes. A total ion chromatogram (TIC) and a representative extracted ion chromatogram (EIC) of the calibration solution and the plant extract are presented in Figures 2 and 3 for caffeic acid, protocatechuic acid, 4-*O*-caffeoylquinic acid, and 3,5-di-*O*-caffeoylquinic acid.

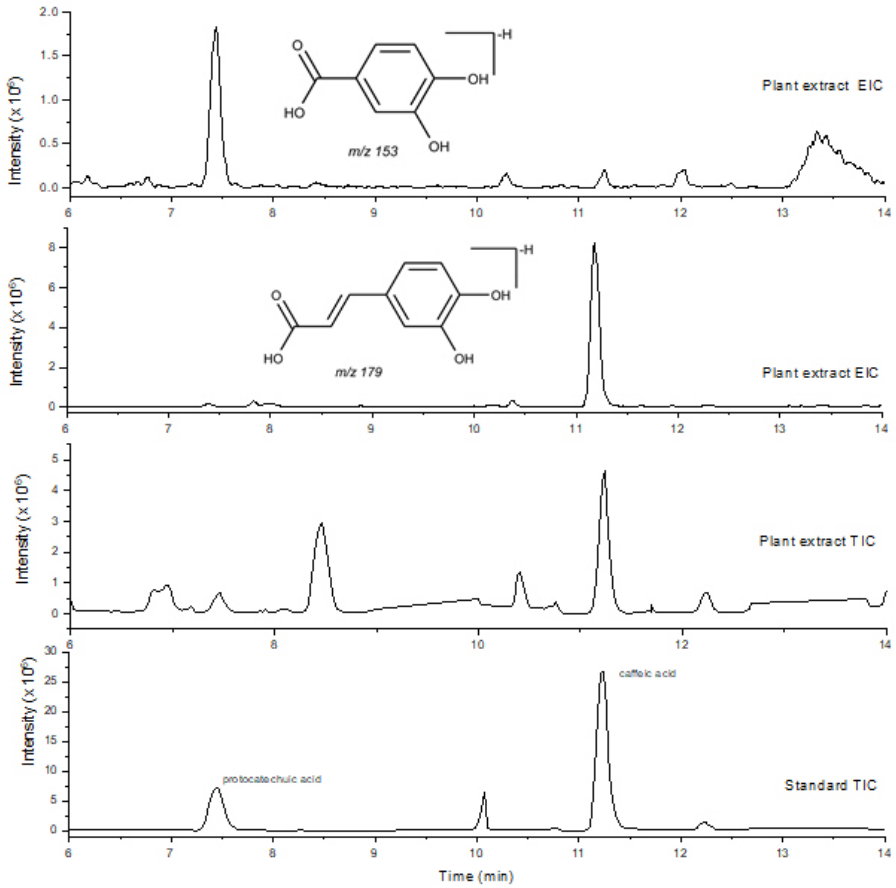

Figure 2. Protocatechuic acid and caffeic acid from the aqueous-methanolic extract of *Xanthium spinosum* aerial part: total ion chromatogram (TIC) and

extracted ion chromatogram (EIC) of the calibration solution and the plant extract

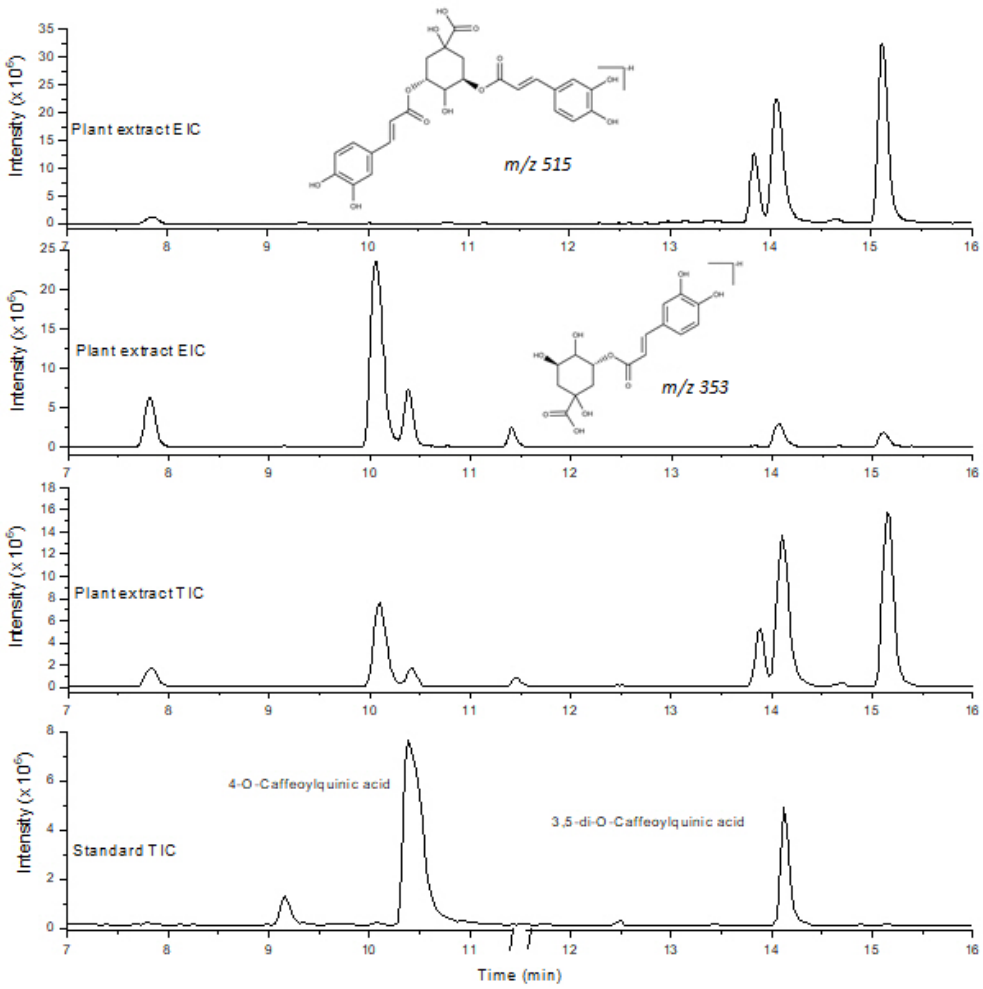

Figure 3. 4-*O*-Caffeoylquinic acid, and 3,5-di-*O*-caffeoylquinic acid from the aqueous-methanolic extract of *Xanthium spinosum* aerial part: total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of the calibration solution and the plant extract

3. Conclusions

HPLC-ESI-MS/MS was applied for the analysis of the metabolite profile of aerial parts of *Xanthium spinosum*. Protocatechuic acid, 4-*O*-caffeoylquinic acid, and 3,5-di-*O*-caffeoylquinic acid are reported for the first time in *X. spinosum*. Further analyses are necessary to establish the role of the detected compounds in the biological activities, including the anti-inflammatory and antioxidant effects of *X. spinosum*.

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