THE INVESTIGATION OF BIOACTIVE SECONDARY METABOLITES OF THE METHANOL EXTRACT OF ERYNGIUM AMETHYSTINUM

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(Received June 22, 2017; Accepted September 10, 2017)

ABSTRACT. Eryngium amethystinum L. belonging to the Apiaceae family, is a perennial plant distributed in Southeast Europe. Even though this plant is used in traditional medicine, its phytochemical characterization is still incomplete. In this study composition of bioactive constituents of the methanol extract are reported for the first time. By means of the UPLC-LTQ-Orbitrap-MS method, altogether sixty-three constituents were characterized: eight hydroxybenzoic acid derivatives (7-13, 32), fifteen cinnamic acid derivatives (14, 17-19, 21, 24-26, 28, 30, 39-42 and 44), four flavonoid aglycones (45, 51, 52, 54), twenty-four flavonoid derivatives (23, 27, 29, 31, 33-38, 43, 46-50, 53, 55-59, 61 and 62), three coumarin derivatives (15, 16 and 22) and nine other compounds (1-6, 20, 60 and 63).

Keywords: metabolic profiling, LC-HRMS, phenolic compounds.

INTRODUCTION

The genus Eryngium L. is distributed all around the world, and with more than 200 species represents the taxonomically most complex genus of the family Apiaceae (Worz, 2004; Calvino et al., 2007). Many plants from this genus have valuable ethnopharmacological and nutritional values (Facciola, 1990; Zhang et al., 2008).

Eryngium amethystinum L., commonly known as amethyst sea holly, is distributed in western and central Serbia on calcareous and arid soil, up to 1600 m above the sea level. In folk medicine of Southeast Europe E. amethystinum is used for its diuretic and laxative
properties, in treatment of edemas, urinary ailments, and acidosis. It is also useful as an aid to digestion (FLAMINI et al., 2008).

Previous chemical investigations of Eryngium species revealed the presence of various class of natural products: polyphenols, coumarins, saponins, acetylenes, essential oils (CROWDEN et al., 1969; DRAKE and LAM, 1972; KARTAL et al., 2006; FLAMINI et al., 2008; ZHANG et al., 2008).

Linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) delivers single-stage mass analysis providing molecular mass information, two-stage mass analysis (MS\(^2\)) and multi-stage mass analysis (MS\(^n\)) delivering structural information. Exact mass measurements and elemental composition assignment are essential for the characterization of small molecules. Accurate mass measurement of the product ions formed in MS\(^n\) experiments facilitates the elucidation of the structures of unknown compounds (TCHOUMTCHOUA et al., 2013).

The aim of the present study was to characterize the secondary bioactive metabolites in the methanol extract of E. amethystinum by using UPLC-HRMS\(^n\). The chemical structures of the identified compounds and their glycoside derivatives are important to reveal information on possible bioactive effect of examined plant.

In this paper we presented for the first time chemical profiling and levels of antioxidant activity of the methanol extracts of wild growing E. amethystinum.

**MATERIALS AND METHODS**

**Chemicals and reagents**

LC-MS grade formic acid and acetonitrile were purchased from Thermo Scientific Pierce (Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Brea, CA, USA).

**Plant material and sample preparation**

Eryngium amethystinum aerial parts were collected (Jun 2014) in the region of Raška (south Serbia, altitude 350 m, 43°30’53’’N, 20°37’07’’E) and identified at the Department of Biology and Ecology, Faculty of Science in Kragujevac, University of Kragujevac (Serbia). Voucher specimen (17088, BEOU) was deposited at the Department of Botany, Faculty of Biology, University of Belgrade, Serbia.

The collected plant material was air-dried in darkness at ambient temperature. A portion of the sample (20 g) was ground to a thin powder, and extracted three times (at room temperature, 24h) with HPLC grade methanol (200 mL). Methanol extracts were combined and evaporated under reduced pressure by means of rotary evaporator at 40 °C. A portion of crude extract (50 mg) was dissolved in methanol, filtered through a membrane filter and used directly for LC-MS analysis.

**UPLC-LTQ-Orbitrap-MS for metabolomic analysis**

Chromatographic separations were performed using an ultrahigh-performance liquid chromatography (UPLC) system consisting of a quaternary Accela 600 pump and Accela Autosampler (ThermoFisher Scientific, Bremen, Germany). Analytical column used for separations was a Synchros C\(_{18}\) column (100 x 2.1 mm, 1.7 µm particle size, ThermoFisher Scientific). The mobile phase consisted of (A) ultrapure water with 1% formic acid and (B)
acetonitrile (MS grade). The injection volume for the sample was 10 µL, elution gradient programme was 5-95 % B for 20 min, with the flow rate of 0.3 mL/min.

The UPLC system was coupled to a linear-trap quadrupole (LTQ) orbitrap hybrid mass spectrometer equipped with a heated-electrospray ionisation probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). The mass spectra were obtained in negative and positive ion modes. Operation parameters were as follows: source voltage 4.5 kV (4.2 kV in positive mode), capillary voltage -10 V (42 V in positive mode), tube lens voltage -35 V (110 V in positive mode), capillary temperature 300 ºC, sheath and auxiliary gas flow (N\textsubscript{2}) 32 and 8 (arbitrary units), respectively. The MS spectra were acquired by full range acquisition covering 100-1000 m/z, and for the fragmentation study, a data dependent scan was performed by deploying collision-induced dissociation (CID): the normalized collision energy of the CID cell was set to 35 eV.

Compounds were identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. ThermoFisher Scientific Xcalibur software (Version 2.1) was used for instrument control, data acquisition and data analysis.

RESULTS AND DISCUSSION

\textbf{UPLC-LTQ-Orbitrap-MS\textsuperscript{a} identification of secondary metabolites of E. amethystinum}

In the present work, a total of sixty-three compounds were tentatively identified in the sample, mainly phenolic compounds such as: simple phenols, flavonoids, coumarins and other compounds. UPLC-LTQ-Orbitrap MS\textsuperscript{a} chromatograms were given in Figure 1 and Figure 2. The tentatively identified metabolites are summarized in Table 1, in negative and positive ionization modes, including retention times, experimental and calculated [M-H]\textsuperscript{-} and [M+H]\textsuperscript{+}, errors in ppm, MS\textsuperscript{2} and MS\textsuperscript{3} fragment ions (in negative and positive modes), molecular formula, together with their proposed identities. The compounds were identified by interpreting their mass spectra obtained via LTQ-Orbitrap high resolution mass spectrometer and taking into account the data reported in the literature.
Figure 1. UPLC-LTQ-Orbitrap MS in negative mode of methanol extract of *E. amethystinum*.

Figure 2. UPLC-LTQ-Orbitrap MS in positive mode of methanol extract of *E. amethystinum*. 
| Peak | Proposed compounds                               | t<sub>r</sub> (min) | Exp. m/z [M-H]/ [M+H]<sup>+</sup> | Calc. m/z [M-H]/ [M+H]<sup>+</sup> | Error (ppm) | MS<sup>2</sup> fragments (%) | MS<sup>3</sup> fragments (%) | Mol. formula |
|------|--------------------------------------------------|---------------------|-----------------------------------|-----------------------------------|-------------|-------------------------------|-----------------------------|--------------|
| 1    | Gluconic acid                                   | 1.09                | 195.0496/ n. o.                    | 195.0505/ 197.0661               | -1.482/ n. o. | 177 (31), 159 (72), 129 (100); 85 (92), 57 (100)/ n. o. | n. o. | C<sub>6</sub>H<sub>12</sub>O<sub>7</sub> |
| 2    | Quinic acid                                     | 1.15                | 191.0554/ n. o.                    | 191.0556/ 193.0712               | 1.965/ n. o. | 127 (100)/ n. o. | 115 (100), 89 (11), 87 (10), 71 (6); 71 (100)/ n. o. | C<sub>7</sub>H<sub>12</sub>O<sub>6</sub> |
| 3    | Malic acid                                      | 1.43                | 133.0133/ n. o.                    | 133.0137/ 135.0293               | 0.979/ n. o. | 115 (100), 89 (11), 87 (10), 71 (6); 71 (100)/ n. o. | n. o. | C<sub>4</sub>H<sub>6</sub>O<sub>5</sub> |
| 4    | Succinic acid                                   | 1.49                | 117.0185/ n. o.                    | 117.0188/ 119.0344               | 2.263/ n. o. | 59 (100)/ n. o. | n. o. | C<sub>4</sub>H<sub>6</sub>O<sub>4</sub> |
| 5    | Hydroquinone-O-glucoside                        | 1.56                | 271.0818/ n. o.                    | 271.0818/ 273.0974               | 1.958/ n. o. | 109 (100)/ n. o. | n. o. | C<sub>12</sub>H<sub>10</sub>O<sub>7</sub> |
| 6    | Hydroxymethylglutaric acid                      | 2.44                | 161.0446/ 163.0594                | 161.0450/ 163.0606               | 1.243/ n. o. | 117 (100)/ n. o. | n. o. | C<sub>6</sub>H<sub>10</sub>O<sub>5</sub> |
| 7    | Glucosyringic acid                              | 3.17                | 359.0975/ n. o.                    | 359.0978/ 361.1135               | 0.632/ n. o. | 197 (100), 179 (23)/ n. o. | n. o. | C<sub>15</sub>H<sub>20</sub>O<sub>10</sub> |
| 8    | 1-Galloyl-O-glucoside                           | 3.25                | 331.0644/ n. o.                    | 331.0665/ 333.0822               | -0.872/ n. o. | 152 (100), 124 (7)/ n. o. | n. o. | C<sub>13</sub>H<sub>10</sub>O<sub>10</sub> |
| 9    | Protocatechuic acid glucoside (isomer)          | 3.35                | 315.0721/ n. o.                    | 315.0716/ 317.0873               | 3.147/ n. o. | 153 (100)/ n. o. | n. o. | C<sub>13</sub>H<sub>10</sub>O<sub>9</sub> |
| 10   | Syringic acid                                   | 3.49                | 197.0447/ n. o.                    | 197.0450/ 199.0606               | 1.320/ n. o. | 153 (100)/ n. o. | n. o. | C<sub>9</sub>H<sub>10</sub>O<sub>5</sub> |
| 11   | Protocatechuic acid                             | 3.68                | 153.0185/ n. o.                    | 153.0188/ 155.0344               | 1.862/ n. o. | 109 (100)/ n. o. | n. o. | C<sub>7</sub>H<sub>6</sub>O<sub>4</sub> |
| 12   | 4-(β-D-Glucosyloxy)benzoic acid                 | 3.71                | 299.0755/ n. o.                    | 299.0767/ 301.0923               | -2.153/ n. o. | 137 (100), 93 (27)/ n. o. | n. o. | C<sub>13</sub>H<sub>10</sub>O<sub>8</sub> |
| 13   | 1-O-(4-Hydroxy-3,5-dimethoxybenzoyl)-glucoside  | 4.17                | 359.0966/ 359.0978                | -1.819/ n. o.                    | 197 (100), 182 (3); 182 (100), 153 | | | | C<sub>15</sub>H<sub>20</sub>O<sub>10</sub> |
| No. | Compound                          | Rf  | M/z (accurate mass)       | Isomers          | Intensity patterns                                                                 |
|-----|----------------------------------|-----|---------------------------|------------------|----------------------------------------------------------------------------------|
| 14  | Chlorogenic acid (isomer)         | 4.69| 353.0856/355.1017         | 353.0873/355.1029 | -3.055/-1.911 191 (100), 179 (39), 135 (7); 173 (81), 127 (92), 111 (31), 93 (47), 85 (100)/309 (11), 165 (100), 147 (77), 147 (100) |
| 15  | Esculetin-6-O-glucoside           | 4.72| 339.0710/341.0864         | 339.0716/341.0873 | -0.231/-0.817 177 (100)                                                          |
| 16  | Scopoletin-7-O-glucuronide        | 4.76| 367.0659/369.0805         | 367.0665/369.0822 | -0.145/-3.124 191 (100)                                                          |
| 17  | Chlorogenic acid (isomer)         | 4.86| 353.0855/355.1016         | 353.0873/355.1029 | -3.394/-2.080 191 (100), 179 (<3); 173 (83), 127 (96), 111 (30), 93 (49), 85 (100)/309 (10), 165 (100), 147 (63), 147 (100) |
| 18  | 4-O-Glucosyl-p-coumaric acid      | 4.97| 325.0910/327.1078         | 325.0923/327.1080 | -2.350/-1.150 163 (100)                                                          |
| 19  | Chlorogenic acid (isomer)         | 5.06| 353.0851/355.1019         | 353.0873/355.1029 | -4.612/-1.376 191 (100), 179 (15), 161 (3); 173 (84), 127 (94), 111 (33), 93 (52), 85 (100)/309 (10), 165 (100), 147 (87), 147 (100) |
| 20  | Nonyl-O-maltoside                 | 5.10| 467.2493/469.2637         | 467.2492/469.2649 | 1.373/-1.360 191 (100), 179 (39), 135 (7); 173 (81), 127 (92), 111 (31), 93 (47), 85 (100)/309 (11), 165 (100), 147 (77), 147 (100) |
| 21  | Caffeic acid                      | 5.52| 179.0339/ n. o.           | 179.0344/181.0501 | -0.085/-0.867 135 (100)/175 (100)/177 (100)                                       |
| 22  | (4-Methylumbelliferone)-7-O-glucoside | 5.69 | 337.0924/339.1071         | 337.0923/339.1080 | 1.828/-0.918 463 (6), 301 (100), 300 (32)/n. o.                                |
| 23  | Quercetin-3-O-rutinoside          | 5.72| 609.1461/611.1601         | 609.1456/611.1612 | 1.722/-2.177 463 (6), 301 (100), 300 (32)/n. o.                                |
| 24  | 5-O-p-Coumaroylquinic acid        | 5.76| 337.0911/339.1069         | 337.0923/339.1080 | -2.177/-1.516 191 (20), 163 (100)/n. o.                                         |
| 25  | Feruloylquinic acid (isomer)      | 5.94| 367.1017/369.1176         | 367.1029/369.1186 | -1.848/-1.080 191 (100), 173 (<2); 173 (74), 171 (31), 127 (100), 111 (41), 93 (60), 85 (92)/n. o. |
| No. | Compound                                      | Retention | M/z          | M/z              | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          | M/z          |
|-----|----------------------------------------------|-----------|--------------|----------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 26  | Feruloylquinic acid (isomer)                 | 6.14      | 367.1013/369.1177 | 367.1029/369.1186 | -2.993/      | -0.890       | 191 (100), 173 (5), 173 (72), 127 (100), 111 (54), 93 (51), 85 (87)/n.o.       | C_{17}H_{20}O_{9} |
| 27  | Quercetin-3-O-neohesperidoside               | 6.26      | 609.1452/611.1599 | 609.1456/611.1612 | 0.310/       | -1.229       | 301 (100), 273 (14), 271 (71), 193 (7), 179 (100), 151 (62)/465 (19), 303 (100), 285 (41), 257 (100), 229 (88), 165 (59), 359 (100), 197 (7), 161 (14), 223 (14), 197 (92), 179 (49), 161 (100)/n.o. | C_{27}H_{36}O_{16} |
| 28  | 3'-O-Glucopyranosyl rosinamic acid           | 6.34      | 521.1284/523.1439 | 521.1295/523.1452 | -1.165/      | -1.333       | 447 (17), 429 (49), 285 (100); 255 (100), 227 (10)/449 (17), 287 (100), 258 (42), 241 (100), 213 (72), 165 (94), 153 (53) | C_{24}H_{26}O_{13} |
| 29  | Kaempferol-3-O-neohesperidoside              | 6.36      | 593.1500/595.1651 | 593.1506/595.1663 | -0.129/      | -1.086       | 447 (17), 429 (49), 285 (100); 255 (100), 227 (10)/449 (17), 287 (100), 258 (42), 241 (100), 213 (72), 165 (94), 153 (53) | C_{27}H_{36}O_{15} |
| 30  | p-Coumaric acid                              | 6.38      | 163.0388/n.o.       | 163.0395/165.0552 | -1.292/      | n.o.         | 191 (100), 173 (5), 173 (72), 127 (100), 111 (54), 93 (51), 85 (87)/n.o.       | C_{9}H_{8}O_{3}  |
| 31  | Quercetin-3-O-glucoside                      | 6.47      | 463.0859/465.1021   | 463.0877/465.1033 | -2.510/      | -1.446       | 301 (100), 271 (31), 255 (22), 179 (100), 150 (49)/303 (100), 285 (41), 257 (100), 247 (31), 229 (63), 165 (48) | C_{21}H_{20}O_{12} |
| 32  | Methyl 4-hydroxybenzoate                     | 6.52      | 151.0390/153.0540   | 151.0395/153.0552 | 0.128/       | -4.251       | 136 (100), 108 (23), 92 (100)/n.o.         | C_{9}H_{8}O_{3}  |
| 33  | Kaempferol-O-ramnoside-O-xylloside           | 6.55      | 563.1401/565.1543   | 563.1401/565.1557 | 0.973/       | -1.560       | n.o. / 433 (100), 419 (13), 287 (11), 287 (100) | C_{26}H_{26}O_{14} |
| 34  | Kaempferol-3-O-glucoside                     | 6.57      | 447.0927/449.1072   | 447.0927/449.1084 | 1.235/       | -1.465       | 285 (100)/287 (100) | C_{21}H_{20}O_{11} |
| 35  | Isorhamnetin-O-rutinoside                    | 6.63      | 623.1614/625.1751   | 623.1612/625.1769 | 1.154/       | -1.921       | 315 (100), 300 (12), 300 (100), 285 (27) | C_{28}H_{25}O_{16} |
| 36  | Isorhamnetin-O-glucoside                     | 6.75      | 477.1013/479.1175   | 477.1013/479.1190 | -3.128/      | -1.905       | 315 (100), 300 (100), 285 (100)| C_{22}H_{24}O_{12} |
| 37  | Syringetin-O-glucoside                       | 6.86      | 507.1127/507.1139   | 507.1139/507.1139 | -1.276/      | n.o.         | 317 (100), 302 (100)| C_{23}H_{24}O_{13} |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 38 | Luteolin-7-O-glucoside | 6.93 | 509.1282 | 509.1295 | -1.566 |
| 39 | Cichoric acid | 6.97 | 447.0903 | 447.0927/449.1065 | -4.289/3.023 |
| 40 | Dicaffeoylquinic acid (isomer) | 7.06 | 515.1191/517.1333 | 515.1190/517.1346 | 1.315/1.455 |
| 41 | Dicaffeoylquinic acid (isomer) | 7.11 | 359.0750/361.0915 | 359.0767/361.0923 | -3.325/-0.759 |
| 42 | Rosmarinic acid | 7.09 | 447.0907/449.1068 | 447.0927/449.1084 | -3.395/-2.422 |
| 43 | Isorhamnetin-O-pentoside | 7.26 | 515.1195/517.1335 | 515.1190/517.1346 | 2.849/-1.107 |
| 44 | Dicaffeoylquinic acid (isomer) | 7.32 | 315.0495/317.0652 | 315.0505/317.0661 | -1.362/-1.322 |
| 45 | Isorhamnetin | 7.34 | 417.0800/419.0967 | 417.0822/419.0978 | -3.916/-1.322 |
| 46 | Kaempferol-3-O-pentoside | 7.47 | 593.1293/595.1437 | 593.1295/595.1452 | 0.511/-1.474 |
| 47 | Kaempferol-3-O-(p-coumaroyl)-glucoside (isomer) | 8.19 | 623.1370/625.1541 | 623.1401/625.1557 | -4.128/-1.715 |
| 48 | Kaempferol-3-O-(feruloyl)-glucoside (isomer) | 8.29 | 285.0396/287.0547 | 285.0399/287.0556 | 0.756/-1.325 |
| 49 | Kaempferol-3-O-(feruloyl)-glucoside (isomer) | 8.34 | 285.0396/287.0547 | 285.0399/287.0556 | 0.756/-1.325 |
| 50 | Kaempferol-3-O-(p-coumaroyl)-glucoside (isomer) | 8.38 | 593.1293/595.1437 | 593.1295/595.1452 | 0.511/-1.474 |
| 51 | Luteolin | 8.46 | 285.0396/287.0547 | 285.0399/287.0556 | 0.756/-1.325 |
|    | Compound                                    | Rf  | m/z     | m/z     | m/z     | m/z     | m/z     | m/z     | m/z     |            |            |            |            |            |            |
|----|---------------------------------------------|-----|---------|---------|---------|---------|---------|---------|---------|------------|------------|------------|------------|------------|------------|
| 52 | Quercetin                                   | 8.52| 301.0336/303.0493 | 301.0348/303.0505 | -2.156/2.109 | 179 (100), 151 (63), 121 (12)/n. o. | C_{15}H_{16}O_{7} |
| 53 | Quercetin-3-O-(di-p-coumaroyl)-glucoside (isomer) | 9.35| 755.1666/757.1748 | 755.1612/757.1769 | 7.904/-1.996 | n. o. /455 (100), 437 (12); 437 (56), 291 (14), 147 (100) | C_{39}H_{32}O_{16} |
| 54 | Kaempferol                                  | 9.50| 285.0390/287.0547 | 285.0399/287.0556 | -1.384/-0.922 | 257 (11), 229 (100), 213 (67), 151 | C_{15}H_{16}O_{6} |
| 55 | Quercetin-3-O-(di-p-coumaroyl)-glucoside (isomer) | 9.57| n. o. /757.1752 | n. o. /757.1769 | -1.384/-0.922 | n. o. /455 (100), 437 (15); 437 (62), 291 (16), 147 (100) | C_{39}H_{32}O_{16} |
| 56 | Kaempferol-3-O-(di-p-coumaroyl)-glucoside (isomer) | 9.73| 739.1678/741.1803 | 739.1663/741.1819 | 2.765/-1.480 | n. o. /455 (100), 437 (8), 287 (<3); 437 (39), 147 (100) | C_{39}H_{32}O_{15} |
| 57 | Kaempferol-3-O-(p-coumaroyl)-(feruloyl)-glucoside (isomer) | 9.83| 769.1747/771.1909 | 769.1769/771.1925 | -2.043/-1.428 | n. o. /485 (100), 467 (9); 467 (52), 177 (100), 147 (19) | C_{40}H_{34}O_{16} |
| 58 | Kaempferol-3-O-(di-p-coumaroyl)-glucoside (isomer) | 9.92| 739.1695/741.1799 | 739.1663/741.1819 | 5.064/-1.979 | n. o. /455 (100), 437 (9), 287 (11); 437 (32), 147 (100) | C_{39}H_{32}O_{15} |
| 59 | Kaempferol-3-O-(p-coumaroyl)-(feruloyl)-glucoside (isomer) | 10.02| 769.1746/771.1906 | 769.1769/771.1925 | -2.277/-1.830 | n. o. /485 (100), 467 (9); 467 (32), 177 (100), 147 (16) | C_{40}H_{34}O_{16} |
| 60 | Dihydroxyhexadecanoic acid                  | 10.07| 287.2208/289.2368 | 287.2222/289.2379 | -2.980/-1.680 | n. o. /271 (100), 253 (11); 253 (100), 235 (54) | C_{16}H_{32}O_{4} |
| 61 | Kaempferol-3-O-(di-p-coumaroyl)-glucoside (isomer) | 10.12| 739.1685/741.1798 | 739.1663/741.1819 | 3.671/-1.587 | n. o. /455 (100), 437 (8), 287 (11); 437 (32), 147 (100) | C_{39}H_{32}O_{15} |
| 62 | Isorhamnetin-O-(p-coumaroyl)-(feruloyl)-glucoside | 10.18| n. o. /801.2009 | 799.1874/801.2031 | -2.067 | n. o. /485 (100), 467 (5); 467 (49), 177 (100) | C_{41}H_{36}O_{17} |
| 63 | Hexadecanedioic acid                        | 10.49| n. o. /287.2211 | 285.2066/287.2222 | n. o. /-2.040 | n. o. /269 (100); 251 (100) | C_{16}H_{30}O_{4} |
Benzoic acid and hydroxycinnamic derivatives

As can be seen from Table 1, twenty-three compounds were identified. Among them, eight hydroxybenzoic acid and fifteen cinnamic acid derivatives were found. Compound 7 gave a \([M-H]\) ion at \(m/z\) 359 and its MS\(^2\) fragmentation resulted in fragment ions at \(m/z\) 197 [M-glucose] and \(m/z\) 179 [M-glucose-H\(_2\)O], suggesting glucosyringic acid. Syringic acid (10) was identified on the basis of \([M-H]\) ion at \(m/z\) 197 and characteristic loss of CO\(_2\) during formation of MS\(^2\) ion at \(m/z\) 153. Compound 8 exhibited an \([M-H]\) ion at \(m/z\) 331, and its fragmentation showed base peak ion at \(m/z\) 152 [M-glucose-OH]. This compound corresponds to a 1-galloyl-O-glucoside. Protocatechuic acid glucoside, as compound 9, yielded a \([M-H]\) ion at \(m/z\) 315 and its MS\(^2\) fragmentation gave the aglycone fragment ion at \(m/z\) 153 (-162 Da, loss of glucose residue). Elimination of 44 Da from deprotonated molecular ion and MS\(^2\) ion at \(m/z\) 315, indicated that compound 11 was protocatechuic acid. Compound 12, 4-(\(\beta\)-D-glucosyloxy)benzoic acid, was characterized by loss of 162 Da from \([M-H]\) ion at \(m/z\) 299, as well as from MS\(^3\) ion at \(m/z\) 93 [M-glucose-CO\(_2\)]\(^{-}\). After examination of MS\(^2\) (ion at \(m/z\) 197, loss of glucose residue) and MS\(^3\) spectra (ion at \(m/z\) 182, loss of methyl group; ion at \(m/z\) 153, loss of CO\(_2\)), compound 13 was assigned as 1-O-(4-hydroxy-3,5-dimethoxybenzoyl)-glucoside. The molecular ions in negative and positive modes indicated that compound 32 had molecular formula C\(_8\)H\(_8\)O\(_3\). Loss of methyl group during formation of MS\(^2\) ion at \(m/z\) 136, and further elimination of CO\(_2\) during formation of a base peak ion at \(m/z\) 92 in MS\(^3\) spectra, suggested that peak 32 belonged to methyl 4-hydroxybenzoate.

Observed deprotonated molecular ion at \(m/z\) 179 and MS\(^2\) ion at \(m/z\) 135 (loss of 44 Da) indicated that compound 21 was caffeic acid. On the basis of the obtained values of \([M-H]\) and \([M+H]\)^{+}, as well as MS\(^2\) and MS\(^3\) fragmentation patterns (in both negative and positive modes, respectively), compounds 14, 17 and 19 were assigned as isomers of caffeoyl quinic acid or chlorogenic acid. All these compounds showed a negative product ion at \(m/z\) 179 due to loss of a deprotonated molecule of caffeic acid, as well as positive product ion at \(m/z\) 165 due to loss of quinic acid residue (Misić et al., 2015). Comparing MS\(^2\) fragmentation patterns obtained in negative ionization mode with previously published data (Misić et al., 2015; Gardana et al., 2007; Gouveia and Castilho, 2011), three isomeric dicaffeoylquinic acids (compounds 40, 41 and 44) and rosmarinic acid (compound 42) were identified in our sample. The analysis in the LTQ-Orbitrap confirmed presence of 40, 41 and 44 with \([M-H]\) errors of 1.315 ppm, 2.034 ppm and 2.849 ppm, respectively. The ion at \(m/z\) 353 or \([M-H-162]\) corresponded to loss of a caffeic acid unit, while ion at \(m/z\) 191 indicated deprotonated quinic acid. Compound 39 (t, 6.97 min) gave an \([M-H]\) ion at \(m/z\) 473 (error -1.886 ppm). The MS\(^2\) fragmentation gave a fragment ion at \(m/z\) 311 (loss of 162 Da) as deprotonated caftaric acid, base peak ion at \(m/z\) 293 (neutral loss of caffeic acid) and ion at \(m/z\) 179, which corresponds to deprotonated caffeic acid (Figure 3 and Figure 4). Thus compound 39 was characterized as cichoric acid.
Figure 3. The MS$^2$ spectra in negative mode of cichoric acid.

Figure 4. Possible fragmentation pathways of cichoric acid.
Peak 28 with [M-H]− at m/z 521 (error -1.165 ppm) and MS² ion at m/z 359 as deprotonated compound 42 [M-H-glucose]− was characterized as 3′-O-glucopyranosyl rosmarinic acid. By showing [M-H]− ion at m/z 325, as well as MS² ion at m/z 163 (-162 Da glucosyl residue), compound 18 was identified as 4-O-glucosyl-p-coumaric acid. Compounds 30 (p-coumaric acid) and 24 (5-O-p-coumaroylquinic acid) exhibited [M-H]− at m/z 163 and at m/z 337, respectively. The typical loss of CO₂ was observed for compound 30 (MS² base peak ion at m/z 119), while compound 24 in the MS² spectra revealed peaks at m/z 191 and 163 corresponding to the deprotonated quinic acid [M-H-146]− and coumaric acid [M-H-174]−, respectively. Two isomeric feruoylquinic acids (compounds 25 and 26) with [M-H]− at m/z 337 were detected at t_r 5.76 min and t_r 5.96 min, respectively. In both cases, MS² of m/z 337 showed a base ion peak at m/z 191 corresponding to the formation of deprotonated quinic acid.

**Flavonoid aglycones and their derivatives**

Among twenty-eight compounds, four aglycones and twenty-four flavonoid derivatives were identified in methanol extract of _E. amethystinum_.

Peak 51 (t_r 8.46 min, [M-H]− at m/z 301, error 0.756 ppm) was identified as luteolin. Observed fragment ions are at m/z 241 [M-H-CO₂], 199 [M-H-C₃H₂O-CO₂] and 175 [M-H-C₃O₂-C₂H₂O]. Compound 52 (quercetin) yielded an [M-H]− at m/z 301, while MS² fragmentation gave fragment ions from retro-Diels-Alder (RDA) reaction at m/z 179 [1^{2}A]−, 151 [1^{2}A-CO]− and 121 [1^{2}B]− (Fabre _et al._, 2001). Flavonol kaempferol was identified as peak 54 at t_r 9.50 min, by comparing literature data with deprotonated ion at m/z 285, as well as MS² ions at m/z 257 [M-H-CO], 229 [M-H-2CO], 213 [M-H-CO₂-CO] and 151 [1^{2}A]− (Fabre _et al._, 2001). Presence of isorhamnetin (compound 45) was confirmed by its MSⁿ spectra in positive ionization mode. MS² of [M+H]⁺ at m/z 317 (error -1.322 ppm) showed peak at m/z 302, which corresponds to loss of methyl group (CH₃). Further fragmentation showed ions at m/z 301 (loss of H), 274 [M+H-CH₃-CO]⁺, 256 [M+H-CH₃-CO- H₂O]⁺ and 228 [M+H-CH₃-C₂O₂H-OH]⁺, as well as two RDA fragmentation ions at m/z 152 [1^{3}A]⁺ and 124 [1^{3}A]⁺.

Three quercetin glycosides 23, 27 and 31 were found in our sample. Quercetin-3-O-rutinoside (23) with an error of 1.722 ppm yielded [M-H]− ion at m/z 609. The MS² fragmentation gave ions at m/z 463 (loss of 164 Da, rhamnosyl residue) and aglycone fragment ion of deprotonated quercetin (Y_O) at m/z 301 (loss of 162 Da, glucose residue). Compound 27, quercetin-3-O-neohesperidoside, was observed both in negative and positive ionization modes, respectively. The molecular ions were at m/z 609/611 (error 0.310 ppm/-1.229 ppm). MS² in negative mode showed ion at m/z 301 (loss of 308 Da, sugar residue), while MS³ fragment ions confirmed presence of quercetin moiety. Further, presence of neohesperidoside residue was confirmed by MS² fragment ions in positive mode. Observed ion at m/z 465 corresponds to quercetin-3-O-glucoside (-146 Da indicated on rhamnose part), while base peak ion at m/z 303 was protonated quercetin (Y_O⁺) (-308 Da, neohesperidoside residue). Quercetin-3-O-glucoside (compound 31) with [M-H]/[M+H]⁺ at m/z 463/465 (error -2.510 ppm/-1.446 ppm) gave origin to quercetin aglycone, by loss of glucose moiety (m/z 301 in negative mode; m/z 303 in positive mode) (Aaby _et al._, 2012). Two quercetin derivatives (compounds 53 and 55) with observed [M+H]⁺ ions at m/z 757 (errors -1.996 ppm and -1.507 ppm, respectively) and similar MS² and MS³ spectra were identified as quercetin-3-O-(di-p-coumaroyl)-glucoside isomers. The MS² mass spectra showed base peak ion at m/z 455, which corresponds to loss of quercetin [M+H-302]⁺ and ion at m/z 437 [M+H-quercetin-
H$_2$O$^+$. The MS$^3$ mass spectra displayed ion at $m/z$ 437, indicating neutral loss of H$_2$O, ion at $m/z$ 291 which corresponds to coumaroyl glucoside residue (loss of 164 Da or $p$-coumaric acid), and ion at $m/z$ 147 which confirmed presence of coumaroyl moiety (Figure 5).

Figure 5. The MS$^2$ and MS$^3$ spectra in positive mode of quercetin-3-O-(di-$p$-coumaroyl)-glucoside.

Compound 29 exhibited an [M-H]/[M+H]$^+$ at $m/z$ 593/595 (error -0.129 ppm/-1.086 ppm). The presence of neohesperoside residue was confirmed by MS$^2$ fragment ions. Observed ions at $m/z$ 447/449 correspond to kaempferol-3-O-glucoside (-146 Da indicated on rhamnose part), while base peak ions at $m/z$ 285/287 correspond to kaempferol residue (loss of 308 Da, neohesperidoside residue). Compound 33 gave [M+H]$^+$ ion at $m/z$ 565 and in the
MS² fragmentation a neutral loss of 146 Da (rhamnosyl residue) was observed to produce a fragment ion at m/z 419. Similarly, loss of xylosyl residue (-132 Da) during formation of ion at m/z 433 was found. With the facts that MS² and MS³ spectra showed protonated ions from kaempferol, and that under used experimental condition it was not possible to determine substitution of flavonoid hydroxyl groups, compound 33 was tentatively identified as kaempferol-O-rhamnoside-O-xylloside. Compound 34, kaempferol-3-O-glycoside, displayed [M-H]⁺/[M+H]⁺ ions at m/z 447/449, as well as characteristic MS² fragmentation ions (in negative and positive modes) at m/z 285/287 (-162 Da, loss of glucose residue) (Ornelas-Paz et al., 2013). Another kaempferol glycoside (compound 46) was tentatively identified as kaempferol-3-O-pentoside. Its [M-H]⁺/[M+H]⁺ ions were observed at m/z 417/419 (error, -3.916 ppm/-1.344 ppm), while neutral loss of 132 Da during formation of MS² fragment ions at m/z 285/287 indicated pentose residue. Compounds 47 (t 8.19 min) and 50 (t 8.38 min) exhibited the same [M+H]⁺ at m/z 595 (errors, -1.575 ppm and -1.474 ppm, respectively). The MS² fragmentation lead to a base peak ion at m/z 287 due to loss of 308 Da which was characterized as a combined loss of 162 Da + 146 Da proved by the [M+H-146]⁺ ion at m/z 449. The high retention time of these compounds suggests that the neutral loss of 146 Da is due to a coumaroyl group rather than a rhamnose unit. The ion at m/z 287 corresponds to the protonated aglycone ion of kaempferol, while intense fragment ion at m/z 309 was assigned as coumaroyl-O-glucoside part (HANHINEVA et al., 2008). Compounds 48 and 49, kaempferol-3-O-(feruloyl)-glucoside isomers, showed protonated ion of ferulic acid at m/z 195, as well as ion of kaempferol at m/z 287. Again, MS² fragment ion at m/z 339 belongs to feruloyl-O-glucoside. Three kaempferol-3-O-(di-p-coumaroyl)-glucoside isomers (compounds 56, 58 and 61) were observed at t 9.73 min, 9.92 min and 10.12 min, respectively. MS² fragment ion at m/z 287 indicated presence of kaempferol. The MS² base peak ion at m/z 455 [M+H-kaempferol]⁺ corresponds to the di-p-coumaroyl-O-glucoside residue. The presence of one coumaroyl residue was confirmed by formation of MS³ base peak ion at m/z 147, while neutral loss of 308 Da (coumaroyl-O-glucoside) [M+H-kaempferol-308]⁺ indicated occurrence of other coumaroyl residue. Compounds 57 and 59 gave the same [M+H]⁺ at m/z 771 (errors, -1.428 ppm and -1.830 ppm, respectively). The MS³ spectra showed base peak ion at m/z 485 [M+H-286]⁺, indicating the neutral loss of kaempferol. Further fragmentations of ion at m/z 485 lead to the formation of MS³ ions at m/z 177 (-308 Da, coumaroyl-O-glucoside part) and m/z 147 (-338 Da, feruloyl-O-glucoside part), indicating occurrence of feruloyl and coumaroyl residues, respectively. Thus, those two isomers were identified as kaempferol-3-O-(p-coumaroyl)-(feruloyl)-glucosides. Compound 35 exhibited [M-H]⁻ ion at m/z 623 (error 1.154 ppm) showing base peak at m/z 315 (isorhamnetin aglycone), by loss of rutinoside residue (-308 Da). Hence, this compound was tentatively identified as isorhamnetin-O-rutinoside. Compounds 36 and 43 showed [M-H]⁻/[M+H]⁺ ions at m/z 477/479 and 447/449, respectively, and losses of different sugar moieties (-162 Da, glucoside residue; -132 Da, pentoside residue) resulting in isorhamnetin aglycone (m/z 315/317) (Rivera-Pastrana, Yahia, & Gonzalez-Aguilar, 2010). Compound 62 gave [M+H]⁺ ion at m/z 801 (error -2.067 ppm). The MS² spectra showed base peak ion at m/z 485 (coumaroyl-feruloyl-O-glucoside), corresponding to the loss of isorhamnetin. Further fragmentations lead to the formation of ion at m/z 467 [M+H-isorhamnetin-H₂O]⁺. MS³ base peak ion at m/z 177 corresponds to the feruloyl residue, while neutral loss of 308 Da confirmed presence of coumaroyl-O-glucoside part. Thus, compound 62 was identified as isorhamnetin-O-(p-coumaroyl)-(feruloyl)-glucoside.

Compound 37 exhibited an [M+H]⁺ ion at m/z 509 (error -1.566). The MS² fragmentation revealed a neutral cleavage of 162 Da (glucoside residue), leading to the fragment ion at m/z 347, which corresponds to the protonated molecule of syringetin. Thus, this compound was identified as syringetin-O-glucoside. Compound 38 was characterized as
luteolin-7-O-glucoside. This compound showed an [M-H]⁻ ion at m/z 447 and the MS² experiment resulted in a neutral loss of 162 Da (ion at m/z 285) and typical fragment of luteolin.

**Coumarin derivatives**

Compound 15 (esculetin-6-O-glucoside, tᵣ 4.72 min) displayed a deprotonated molecular ion at m/z 339 and MS² fragmentation gave a neutral fragment of 162 Da (glucoside residue), forming the aglycone ion (Yₒ) at m/z 177 (esculetin). Scopoletin-7-O-glucuronide was identified as compound 16; [M-H]- at m/z 367 (error of -0.145 ppm). The MS² spectra showed deprotonated ion of scopoletin (m/z 191), while neutral loss of 176 Da corresponded to the glucuronide residue. (4-Methylumbelliferone)-7-O-glucoside (compound 22) was identified in negative and positive ionization modes. Molecular ions were observed at m/z 337 (error 1.828 ppm) and at m/z 339 (error -0.867 ppm), respectively. The MS² spectra showed neutral loss of 162 Da, which indicated presence of glucoside residue, while ions at m/z 175/177 confirmed presence of 4-methylumbelliferone.

**Other compounds**

The analysis in the LTQ-Orbitrap confirmed presence of five organic acids (1, 2, 3, 4 and 6). Gluconic acid (compound 1) gave a [M-H]⁻ ion at m/z 195 (error -1.482 ppm) and characteristic MS² fragment ions at m/z 177 [M-H-H₂O]⁻, m/z 159 [M-H-2H₂O]⁻, and m/z 129 [M-H-2H₂O-CH₂O]⁻. Compound 2 with deprotonated molecular ion at m/z 191 and fragment ion at m/z 127 (loss of CO and H₂O) was identified as quinic acid. Peak 3, characterized as malic acid, showed an [M-H]⁻ at m/z 133 (error 0.979 ppm), with an MS² ion at m/z 115 corresponding to the loss of water. Neutral loss of C₂H₂O₂ (m/z at 59) from the [M-H]⁻ at m/z 117 confirmed presence of succinic acid (compound 4). Peak 6, with a [M-H]⁻ at m/z 161 (error 1.243 ppm), was identified as hydroxymethylglutaric acid, and its MS² spectra showed ion at m/z 117 (loss of 44 Da, CO₂). Compound 5 corresponded to hydroquinone-O-glucoside ([M-H]⁻ at m/z 271) with a fragmentation pattern at m/z 109 [M-H-glucose]. Compound 20, which showed protonated molecular ion at m/z 469 (error -1.360 ppm), was tentatively identified as nonyl-O-maltoside. MS² spectra revealed loss of H₂O (m/z 451) and glucoside residue (m/z 307). On the basis of observed [M+H]⁺ ions, as well as MSⁿ spectra, compounds 60 and 63 were characterized as dihydroxyhexadecanoic acid and hexadecanedioic acid, respectively.

**CONCLUSIONS**

In this study we performed for the first time UPLC-LTQ-Orbitrap-MSⁿ non-targeted metabolic analysis of methanol extract of *E. amethystinum*. The approach used allowed identification of sixty-three secondary metabolites, most of them belonging to the phenolics class (simple phenolics, flavonoids and coumarins). These data suggest that this medicinal plant might be a valuable source of bioactive secondary metabolites from classes of phenolic acids, flavonoids and coumarins with beneficial proprieties, and a promising source of health products for functional food or nutraceutical industries.
Acknowledgments

This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grants III41010, OI172016 and OI175039). The authors acknowledge the support of the FP7 RegPot project FCUB ERA GA No. 256716. The EC does not share responsibility for the content of the article.

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