PHARMACIA

Pharmacia 71: 1–11
DOI 10.3897/pharmacia.71.e122436

Research Article

# UHPLC-HRMS-based profiling and simultaneous quantification of the hydrophilic phenolic compounds from the aerial parts of *Hypericum aucheri* Jaub. & Spach (Hypericaceae)

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**Received** 6 March 2024 ♦ **Accepted** 8 March 2024 ♦ **Published** 4 April 2024

**Citation:** Marinov T, Kokanova-Nedialkova Z, Nedialkov P (2024) UHPLC-HRMS-based profiling and simultaneous quantification of the hydrophilic phenolic compounds from the aerial parts of *Hypericum aucheri* Jaub. & Spach (Hypericaceae). Pharmacia 71: 1–11. https://doi.org/10.3897/pharmacia.71.e122436

## Abstract

A validated UHPLC-HRMS method was developed to identify and quantify polar phenolic metabolites in the EtOH extract from *H. aucheri* Jaub. & Spach's aerial parts. The external standards, chlorogenic acid, mangiferin, and hyperoside were selected in this analysis. Forty-four compounds, encompassing hydroxybenzoic and hydroxycinnamic acids derivatives, benzophenones, catechins, xanthones, flavonols, biflavones, and chromones were detected and quantified in the aerial parts of the titled plant. Pentahydroxyxanthone-C-glycoside 15, maclurin-O-(benzoyl)-hexoside 37, norathyriol-O-(benzoyl)-hexosides 38 and 42 were suggested to be new natural compounds, while maclurin-O-hexoside 2 was reported for the first time for *Hypericum* genus. Additionally, more than 22 secondary metabolites, including benzophenones, hydroxycinnamic acid derivatives, catechins, and a chromone, were identified for the first time in *H. aucheri*. The amounts of the detected metabolites were calculated relative to external standards. The dominant polar phenolic constituents were chlorogenic acid (11.55 mg/g D.W.) and mangiferin (9.13 mg/g D.W.).

## Keywords

Hypericaceae, flavonols, xanthones, benzophenones, UHPLC-HRMS, quantification

## Introduction

The genus *Hypericum* L. (Fam. Hypericaceae) includes more than 500 species comprising perennial herbaceous plants, shrubs, or small trees, distributed throughout the world, except Antarctica, and avoiding areas of extreme dryness and very high temperature and/or salinity (Crockett and Robson 2011; Robson 2016). *Hypericum aucheri* Jaub. & Spach (sect. *Crossophyllum*) is an herbaceous perennial flowering plant distributed in South-Eastern Bulgaria, Greece (the Aegean Islands and North-East part of the country) as well as in the European and North-Western Anatolian Turkey (Robson 2013). Previous phytochemical studies of the titled plant revealed the presence of xanthones, flavonoids, chlorogenic acid (Kitanov et al. 1979; Kitanov 1988), prenyloxy chromanone derivatives

(aucherine A-C), and prenylated phloroglucinols (Nedialkov et al. 2019). The previous studies of the title plant include developing a densitometric method for the quantitation of mangiferin and isomangiferin (Nedialkov et al. 1998) as well as establishing acute toxicity, anti-depressive and MAO inhibitory activity of the former compound (Dimitrov et al. 2011). In this study, we presented a comprehensive profiling method using LC-MS to analyze hydrophilic phenolic compounds in the aerial parts of *H. aucheri*. Additionally, we developed and validated a UHPLC-HRMS method for simultaneous quantification of the main compounds.

# Material and methods

## Apparatus, materials, and chemicals

The Ultra High-Performance Liquid Chromatograph (UHPLC) Thermo Scientific (Germering, Germany) Dionex UltiMate 3000 RSLC consisted of SRD-3600 solvent degasser, HPG-3400RS high-pressure binary pump, WPS-3000TRS autosampler, and TCC-3000RS thermostatic column compartment. The UHPLC effluent was online connected to a Thermo Scientific (Bremen, Germany) Q Exactive Plus Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. All solvents were of HPLC or LC/MS grade and were purchased from Fisher Scientific (Pittsburgh, USA). Hyperoside, chlorogenic acid, and mangiferin (≥ 97%, HPLC) were purchased from Sigma-Aldrich (Taufkirchen, Germany) or TCI Deutschland GmbH (Eschborn, Germany).

## Plant material

The above-ground parts of *Hypericum aucheri* Jaub. et Spach were gathered from a wild population near Momchilgrad (Kardzali District, Bulgaria) in July 2021. The botanical identity was confirmed by P. Nedialkov. A voucher specimen taken from the population (SOM-Co-1344) was deposited in the herbarium of the Institute of Biodiversity and Ecosystem Research (IBER) at the Bulgarian Academy of Sciences (BAS).

## Extraction and sample preparation

The powdered air-dried aerial parts of *H. aucheri* (250.0 mg) were sonicated at room temperature with *ca* 20 mL 70% EtOH for 30 min and then were diluted to 25 mL with the same solvent. The resulting extract was centrifuged at 15000 rpm for 15 min. One mL aliquot of the supernatant was evaporated to dryness under $N_2$, suspended in 500 µL 1% formic acid in water, and further purified by solid-phase extraction over Phenomenex (Torrance, USA) Strata C18-E (55 µm, 70 Å, 200 mg, 3 mL) cartridge. The sorbent was first washed with $H_2O$ (5 × 500 µL), then eluted with 35% MeCN (10 × 500 µL) in 10.0 mL volumetric flask and diluted to the nominal volume with the same solvent. Subsequently, 1 mL of solution was diluted to 25 mL 35% MeCN. The latter solution was used for qualitative and quantitative analysis of phenolic compounds by UHPLC–ESI-MS/MS.

## UHPLC chromatographic conditions

UHPLC separations were performed on a Nouryon (Göteborg, Sweden) Kromasil C18 column (2.1×100 mm, 1.8 µm) coupled with a precolumn Phenomenex SecurityGuard ULTRA UHPLC EVO C18 at 40 °C. Each chromatographic run was carried out with a binary mobile phase consisting of water containing 0.1% (v/v) formic acid (A) and acetonitrile also with 0.1% (v/v) formic acid (B). A gradient program was used as follows: 0–0.5 min, 5% B; 0.5–3 min, from 5 to 8% B; 3–12 min, from 8 to 15% B; 12–15 min, from 15 to 25% B; 15–24 min, from 25 to 55% B, 24–25 min, from 55 to 95% B, 25–27 min, kept 95% B. Before each run the column was equilibrated for 4.5 min with the initial conditions. The flow rate was 0.3 $mL.min^{-1}$ and the sample injection volume was 2 µL.

## High-resolution electrospray ionization mass spectrometry (HRESIMS) conditions

The experiments were run in negative mode. The tune parameters of the HESI source were as follows: spray voltage −2.5kV; capillary temperature – 320 °C; sheath gas – 38 arbitrary units (a.u.); auxiliary gas – 12 a.u.; probe heater temperature – 320 °C; S-Lens RF Level – 50. The detection and identification of the metabolites were done using a full scan – data-dependent MS/MS (Top 5) experiment. The full scan parameters: resolution, automatic gain control (AGC) target, max. inject time (IT), and mass range were set to 70000 FWHM, $3\times10^6$, 100 ms, and *m/z* 150 to 1000, respectively. The data-dependent MS/MS ($ddMS^2$) parameters were as follows: resolution 17500 FWHM, AGC target $1\times10^5$, max. IT 50 ms, TopN 5, isolation window *m/z* 2.0, stepped NCE 20, 40, 70. The quantitation of phytochemicals in *Hypericum aucheri* was done using full MS/SIM scan experiments. The method parameters were set as follows: resolution 70000 FWHM, AGC target $3\times10^6$, max IT 200 ms, mass range *m/z* 200 to 1000. The selected quantification ions for chlorogenic acid, mangiferin, and hyperoside were at *m/z* 353.0867, 421.0765, and 463.0871, respectively. The mass tolerance was 20 ppm. The data were acquired and processed with Thermo Fisher Scientific Xcalibur ver. 4.1 or FreeStyle ver. 1.8 SP2 QF1.

## Method validation

The quantification of phenolic compounds was carried out using the external standard method. The amount of 44 detected phenolic compounds was calculated relative to external standards of chlorogenic acid, mangiferin, and hyperoside. Each of the external standards (about 5 mg) was dissolved in 20 mL 70 vol. % EtOH (primary solutions).

The stock standard solution of the external standards was prepared by combining the aliquots (1 mL) of each primary solution and dilution to 50 mL with 70 vol. % EtOH. The working standard solutions of appropriate concentration were prepared by diluting the stock standard solution with 70 vol. % EtOH. External standard calibrations were established on six data points covering the concentration range of 16.56–530.00 ng/mL for chlorogenic acid, 16.72–535.00 ng/mL for mangiferin, and 17.97–575.00 ng/mL for hyperoside. The procedure and the parameters of validation were previously described in detail elsewhere (Kokanova-Nedialkova and Nedialkov 2021).

# Results and discussion

## Method validation

In this study, ultra-high performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) was used to detect the polar phenolic compounds in the EtOH extracts from the aerial parts of *Hypericum aucheri* Jaub. et Spach. The efficiency of the extraction procedure and optimization of the chromatographic conditions were as given in the literature (Kokanova-Nedialkova and Nedialkov 2021). Briefly, MeOH, EtOH, i-PrOH, and MeCN as well as their mixtures with water were employed as solvents. The best results were obtained with 70% EtOH. Three chromatographic columns, namely Kromasil C18 column (2.1×100 mm, 1.8 μm), Kromasil Eternity XT C18 column (2.1×100 mm, 1.8 μm), and Phenomenex Kinetex EVO C18 (100×2.1 mm, 1.7 μm) were tested for separation of polar phenolic compounds from the titled plant. The former column showed the best results and was selected for developing the method. Quantitative determination of phenolic compound contents in the aerial parts of *H. aucheri* was performed by the method of the external standard. Chlorogenic acid, mangiferin, and hyperoside were selected as standards for the calculation of the amount of the polar phenolic metabolites. The separation of the standard is given in Fig. 1.

The calibration curves were linear over the concentration range of 16.56–530 ng/mL, 16.72–535 ng/mL, and 17.97–575 ng/mL for chlorogenic acid, mangiferin, and hyperoside, respectively. All calibration curves showed very good linear regressions and the correlation coefficients were $R^2 > 0.999$ (Table 1). The method showed that LODs and LOQs were 0.75 ng/mL and 2.26 ng/mL for chlorogenic acid, 1.11 ng/mL and 3.35 ng/mL for mangiferin, and 0.56 ng/mL and 1.70 ng/mL for hyperoside, respectively (Table 1).

The accuracy of the method was checked by the addition of a standard solution mixture at three concentrations (53.0, 106.00, and 159.00 ng/mL for chlorogenic acid; 65.50, 131.00, and 196.50 ng/mL for mangiferin; 57.50, 115.00 and 172.50 ng/mL for hyperoside) close to that expected in the real plant samples. Blank samples from the

**Table 1.** Linearity of calibration curve for the chlorogenic acid, mangiferin, and hyperoside.

| External standard | Linear range (ng/mL) | Regression equation | $R^2$ | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|
| Chlorogenic acid | 16.56–530.00 | Y = -3692.68+79214.9*X | 0.9998 | 0.75 | 2.26 |
| Mangiferin | 16.72–535.00 | Y = 125990+91573.4*X | 0.9990 | 1.11 | 3.35 |
| Hyperoside | 17.97–575.00 | Y = 178898+127557*X | 0.9996 | 0.56 | 1.70 |

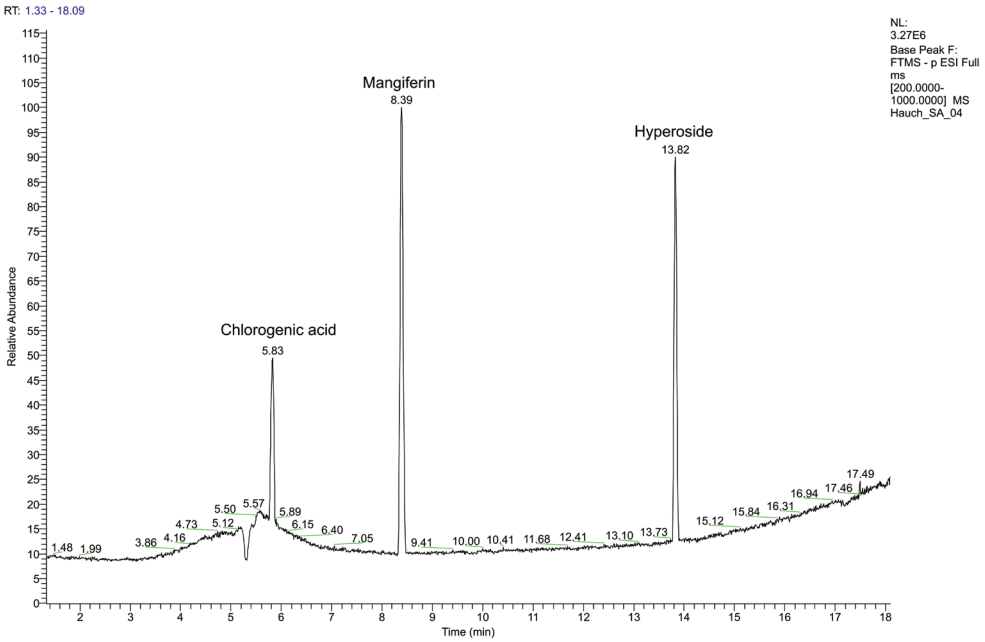


**Figure 1.** Chromatographic separation of the standards chlorogenic acid, mangiferin, and hyperoside under the optimized conditions.

same un-spiked plant extract were analyzed at the same time as the spiked samples and the measured values were subtracted. Furthermore, the related compounds showed overall recoveries ranging from 96.29% to 103.42% with RSD ranging from 0.24% to 2.18%. The method has acceptable accuracy evidenced by the good correlation of the spiked and determined concentrations (Table 2).

**Table 2.** Accuracy of the UHPLC-HRMS method.

| External standard | Added (ng/mL) | Found[a](ng/mL) | Recovery[a] (%) | RSD (%) |
|---|---|---|---|---|
| Chlorogenic acid | 53.00 | 51.13 ± 0.21 | 96.47 ± 0.40 | 0.41 |
| | 106.00 | 107.27 ± 1.67 | 101.20 ± 1.57 | 1.55 |
| | 159.00 | 164.43 ± 1.06 | 103.42 ± 0.67 | 0.65 |
| Mangiferin | 65.50 | 65.42 ± 1.43 | 99.88 ± 2.18 | 2.18 |
| | 131.00 | 132.42 ± 2.12 | 101.08 ± 1.62 | 1.60 |
| | 196.50 | 198.12 ± 0.48 | 100.82 ± 0.25 | 0.24 |
| Hyperoside | 57.50 | 55.37 ± 0.74 | 96.29 ± 1.28 | 1.33 |
| | 115.00 | 112.87 ± 1.30 | 98.15 ± 1.13 | 1.15 |
| | 172.50 | 166.81 ± 2.21 | 96.70 ± 1.28 | 1.33 |

[a] Values are the mean ± SD (n = 3).

The precision of the retention times was estimated by analyzing the repeated runs during a single day and on three different days, respectively. The RSDs of retention times of the standards were ≤ 0.18 % for intra-day and ≤ 0.08 % for inter-day evaluations, respectively (Table 3). For intra-day and inter-day precision tests the evaluated analytes exhibited overall recoveries ranging from 97.40% to 100.46% with RSDs from 0.30% to 1.12%. (Table 3).

The developed UHPLC-HRMS method was applied for the quantification of the polar phenolic compounds detected in the EtOH extract from the aerial parts of *H. aucheri*.

**Table 3.** Evaluation of intra-day (repeatability) and inter-day (intermediate precision) precision of the UHPLC-HRMS method applied on chlorogenic acid, mangiferin, and hyperoside.

| Precision type | RT ± SD (min) | RSD (%) | Recovery ± SD (%) | RSD (%) |
|---|---|---|---|---|
| **Chlorogenic acid** | | | | |
| Intra-day | 5.83 ± 0.011 | 0.18 | 98.40 ± 1.11 | 1.12 |
| Inter-day | 5.83 ± 0.004 | 0.08 | 99.31 ± 0.36 | 0.36 |
| **Magniferin** | | | | |
| Intra-day | 8.39 ± 0.012 | 0.14 | 100.46 ± 0.66 | 0.66 |
| Inter-day | 8.40 ± 0.007 | 0.08 | 100.01 ± 0.38 | 0.38 |
| **Hyperoside** | | | | |
| Intra-day | 13.82 ± 0.012 | 0.09 | 97.77 ± 0.61 | 0.63 |
| Inter-day | 13.83 ± 0.005 | 0.04 | 97.40 ± 0.29 | 0.30 |

## Detection, identification, and quantification of the hydrophilic phenolic metabolites in *H. aucheri*

The identified metabolites and their quantities were listed in Table 4 while the chromatogram of the EtOH extract was given in Fig. 2.

### Hydroxybenzoic acids derivatives

The deprotonated molecule $[M-H]^-$ of compound **1** appeared at *m/z* 329.0880 in the full MS scans. Its MS/MS spectrum showed a product ion at *m/z* 167.03 resulting from a neutral loss of 162 Da, indicative of the presence of an O-linked hexose. Subsequently, the decay of the later product ion produced fragments with *m/z* 123.04, 152.01, and 108.02 that corresponded to a loss of carboxyl (44 Da), methyl (15 Da), and both carboxyl and methyl (59 Da) groups, respectively. This fragmentation was specific to vanillic acid (Barragán-Zarate et al. 2022). Thus, compound **1** was tentatively identified as 1-O-vanilloyl-β-D-glucose and was reported for the first time to occur in *H. aucheri*. The quantity of this metabolite, calculated as chlorogenic acid, was found to be relatively small (139.89 μg/g D.W.).

### Benzophenones

The deprotonated molecule $[M-H]^-$ of compound **2** appeared at *m/z* 423.0935. Its MS/MS spectrum showed a base peak ion at *m/z* 261.04 indicating a loss of a hexose moiety. The fragment at *m/z* 151.00 undergoes neutral loss of $CO_2$ producing an ion at *m/z* 107.01 that is in conformance to the postulated fragmentation pathway of maclurin (Fig. 3) (Berardini et al. 2004). Thus, compound **2** was tentatively identified as a maclurin-O-hexoside (Kaya et al. 2011; Xu et al. 2011). Compound **37** showed a deprotonated molecule $[M-H]^-$ at *m/z* 527.1189. Its MS/MS spectrum shared a similar fragmentation pattern with that of **2**. In addition, it showed product ion at *m/z* 405.08 indicating a neutral loss of 122 Da, which is typical for esterified benzoic acid. Thus, compound **37** was tentatively identified as a maclurin-O-(benzoyl)-hexoside. The quantities of metabolites **2** and **37**, calculated as hyperoside, were 3.36 and 2.27 mg/g D.W., respectively. This class of phenolic compounds was reported here for the first time for the title plant. Furthermore, compound **2** was previously identified only in species of the genus *Gentiana* and *Garcinia* (Marinov et al. 2023), while compound **37** was tentatively determined as a new natural product.

### Hydroxycinnamic acids derivatives

The deprotonated molecules $[M-H]^-$ of compounds **3**, **8**, and **11** appeared at *m/z* ranging from 353.0875 to 353.0879, while **6**, **12** and **19** at *m/z* ranging from 337.0934 to 337.0935. The MS/MS spectrum of **3** produced a base peak at *m/z* 191.06 and secondary peaks at *m/z* 179.03 and 135.04, while **6** showed a base peak at *m/z* 163.04 and secondary peaks at *m/z* 119.05 and 191.06. The product ion at *m/z* 191.06 was indicative of the presence of quinic acid, while the other fragments in MS/MS spectra of **3** and **6** were due to the presence of hydroxycinnamic acid moiety. The MS/MS spectra of compounds **8**, **11**, **12**, and **19** showed a base peak at *m/z* 191.06. Metabolites **12** and **19** produced ions with low intensity at *m/z* 163.04 and 119.05, while **8** and **11** at *m/z* 179.03 and 135.04. The compounds **3**, **6**, **8**, **11,** **12**, and **19** showed similar fragmentation patterns typical for hydroxycinnamoyl quinic acids (Clifford et al. 2003). Thus, compounds **3** and **6** were tentatively identified as 3-O-caffeoylquinic acid and 3-O-*p*-couma-

**Table 4.** The detected and identified polar phenolic compounds as well as their quantity in the EtOH extract from the aerial parts of *H. aucheri*.

| No. | $t_R$ (min) | Compound | Class[1] | Exact Mass | Δppm | Ion type | Molecular Formula | MS/MS product ions (intensity in %) | µg/g D.W. ±SD | Calc.[2] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.81 | vanilloyl glucose | HBA | 329.0880 | 4.06 | $[M-H]^-$ | $C_{14}H_{17}O_9$ | 167.03(100), 108.02(65), 152.01(37), 123.04(21) | 139.89 ±5.56 | C |
| 2 | 3.63 | maclurin-O-hexoside | BEN | 423.0935 | 3.04 | $[M-H]^-$ | $C_{19}H_{19}O_{11}$ | 261.04(100), 151.00(83), 107.01(22) | 3361.57 ±5.16 | H |
| 3 | 3.90 | 3-O-caffeoylquinic acid | HCA | 353.0875 | 2.22 | $[M-H]^-$ | $C_{16}H_{17}O_9$ | 191.06(100), 135.04(60), 179.03(57) | 215.9 ±0.14 | C |
| 4 | 4.97 | (+)-gallocatechin | FLO | 305.0667 | 3.81 | $[M-H]^-$ | $C_{15}H_{13}O_7$ | 125.02(100), 305.06(46), 137.02(29), 109.03(24), 179.03(20), 203.03(3), 151.04(3), 287.05(1) | 121.2 ±0.55 | H |
| 5 | 5.22 | ferulic acid 4-O-hexoside | HCA | 355.1037 | 3.69 | $[M-H]^-$ | $C_{16}H_{19}O_9$ | 134.04(95), 193.05(100), 149.06(30), 178.03(20), | 66.27 ±0.39 | C |
| 6 | 5.35 | 3-O-p-coumaroylquinic acid | HCA | 337.0954 | 4.35 | $[M-H]^-$ | $C_{16}H_{17}O8$ | 163.04(100), 119.05(43), 191.06(8) | 109.16 ±0.50 | C |
| 7 | 5.68 | catechin | FLO | 289.0718 | 4.06 | $[M-H]^-$ | $C_{15}H_{13}O_6$ | 289.07(100), 109.03(69), 245.08(54), 125.02(40), 203.07(27), 137.02(25), 151.02(23), 179.03(15), 271.06(3) | 270.96 ±1.52 | H |
| 8 | 5.79 | 5-O-trans-p-caffeoylquinic acid (chlorogenic acid) | HCA | 353.0876 | 2.48 | $[M-H]^-$ | $C_{16}H_{17}O_9$ | 191.06(100), 179.03(2), 135.04(1) | 11548.9 ±65.83 | C |
| 9 | 7.32 | procianidin B2 | FLO | 577.1364 | 3.99 | $[M-H]^-$ | $C_{30}H_{25}O_{12}$ | 125.02(100), 289.07(67), 407.08(62), 161.02(28), 425.09(18), 137.02(17), 245.08(10) | 109.99 ±85.35 | H |
| 10 | 7.62 | 1-O-feruloyl-β-glucose | HCA | 355.1038 | 4.03 | $[M-H]^-$ | $C_{16}H_{19}O_9$ | 235.06(5), 217.05(10), 193.05(15), 160.02 (60), 175.04(100), 134.04(10), 132.02(25) | 52.31 ±0.69 | C |
| 11 | 8.19 | 5-O-cis-p-caffeoylquinic acid | HCA | 353.0879 | 3.26 | $[M-H]^-$ | $C_{16}H_{17}O_9$ | 191.06(100), 179.03(1), 135.04(1) | 1041.81 ±4.98 | C |
| 12 | 8.26 | 5-O-trans-p-coumaroylquinic acid | HCA | 337.0935 | 4.99 | $[M-H]^-$ | $C_{16}H_{17}O_8$ | 191.06(100), 119.05(7), 163.04(6) | 235.85 ±2.32 | C |
| 13 | 8.33 | mangiferin | XAN | 421.0776 | 2.47 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 301.04(100), 331.05(77), 271.03(36), 259.03(30), 403.07(10) | 9130.57 ±55.63 | M |
| 14 | 8.42 | epicatechin | FLO | 289.0720 | 4.48 | $[M-H]^-$ | $C_{15}H_{13}O_6$ | 289.07(100), 109.03(70), 245.08(50), 125.02(36), 203.07(27), 137.02(25), 151.02(19), 179.03(16), 271.06(3) | 737.71 ±0.42 | H |
| 15 | 8.67 | pentahydroxyxanthone-C-glycoside | XAN | 437.0722 | 1.65 | $[M-H]^-$ | $C_{19}H_{17}O_{12}$ | 317.03(100), 347.04(67), 287.02(14), 275.02(11), 419.06(6) | 181.22 ±0.83 | M |
| 16 | 8.96 | isomangiferin | XAN | 421.0771 | 1.31 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 301.04(100), 331.05(66), 271.03(24), 259.03(17), 403.07 (1) | 151.91 ±2.59 | M |
| 17 | 10.00 | 5-O-feruloylquinic acid | HCA | 367.1039 | 4.07 | $[M-H]^-$ | $C_{17}H_{19}O_9$ | 191.06(100), 134.04(13), 193.05(6) | 152 ±0.24 | C |
| 18 | 10.84 | norathyriol-O-hexoside | XAN | 421.0775 | 2.39 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 259.03(100), 421.08(42), 215.03(10), 187.04(4) | 113.12 ±1.00 | M |
| 19 | 10.94 | 5-O-cis-p-coumaroylquinic acid | HCA | 337.0935 | 4.99 | $[M-H]^-$ | $C_{16}H_{17}O_8$ | 191.06(100), 163.04(2), 119.05(1) | 131.19 ±1.90 | C |
| 20 | 11.03 | Myricetin 3-O-galactoside | FLA | 479.0827 | 3.07 | $[M-H]^-$ | $C_{21}H_{19}O_{13}$ | 316.02(100), 271.03(29), 317.03(24), 287.02(17), 178.99 (5), 137.02 (2) | 1517.02 ±42.29 | H |
| 21 | 11.06 | 1,3,5,6-tetrahydroxyxanthone-O-hexoside | XAN | 421.0775 | 3.71 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 258.02(100), 259.02(11), 213.02(6), 229.01(4), 241.01(1) | 238.01 ±0.55 | M |
| 22 | 11.15 | Myricetin 3-O-glucuronide | FLA | 493.0621 | 1.73 | $[M-H]^-$ | $C_{21}H_{17}O_{14}$ | 317.03(100), 151.00(28), 178.99(23), 137.02(17), 316.02 (4) | 914.58 ±43.39 | H |
| 23 | 11.39 | Myricetin 3-O-glucoside | FLA | 479.0828 | 1.56 | $[M-H]^-$ | $C_{21}H_{19}O_{13}$ | 316.02(100), 271.03(29), 317.03(23), 287.02(15), 178.99 (5), 137.02 (2) | 1220.47 ±42.54 | H |
| 24 | 11.53 | Lancerin | XAN | 405.0825 | 2.21 | $[M-H]^-$ | $C_{19}H_{17}O_{10}$ | 285.04(100), 315.05(29), 255.03(12), 243.03(5), 387.08(2) | 182.25 ±1.66 | M |

| No. | $t_R$ (min) | Compound | Class[1] | Exact Mass | Δppm | Ion type | Molecular Formula | MS/MS product ions (intensity in %) | μg/g D.W. ±SD | Calc.[2] |
|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 12.60 | 1,3,5,6-tetrahydroxyxanthone-O-hexoside | XAN | 421.0775 | 3.59 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 258.02(100), 259.02(13), 241.01(2), 229.01(2), 213.02(2) | 292.54 ±2.80 | M |
| 26 | 13.47 | Myricetin 3-O-rhamnoside | FLA | 463.0881 | 2.23 | $[M-H]^-$ | $C_{21}H_{19}O_{12}$ | 316.02(100), 271.03(27), 317.03(26), 287.02(15), 178.99 (8), 137.02 (3) | 2883.08 ±23.81 | H |
| 27 | 13.80 | Quercetin 3-O-galactoside (hyperoside) | FLA | 463.0880 | 2.03 | $[M-H]^-$ | $C_{21}H_{19}O_{12}$ | 300.03(100), 301.04(55), 271.03(50), 255.03(23), 243.03(14), 151.00(10) | 1907.25 ±30.18 | H |
| 28 | 14.02 | Quercetin 3-O- glucuronide (miquelianin) | FLA | 477.0670 | 1.43 | $[M-H]^-$ | $C_{21}H_{17}O_{13}$ | 301.04(100), 151.00(25), 178.99(11), 107.01(8), 300.03(1) | 3234.45 ±93.46 | H |
| 29 | 14.05 | myricetin-O- hexauronide | FLA | 493.0636 | 4.76 | $[M-H]^-$ | $C_{21}H_{17}O_{14}$ | 317.03(100), 299.02(58), 151.00(36), 178.99(21), 137.02 (10), 316.02 (2) | 92.43 ±0.78 | H |
| 30 | 14.15 | Quercetin 3-O-glucoside (isoquercitrin) | FLA | 463.0881 | 2.23 | $[M-H]^-$ | $C_{21}H_{19}O_{12}$ | 300.03(100), 301.04(61), 271.03(54), 255.03(23), 243.03(15), 151.00(11) | 2267.66 ±6.88 | H |
| 31 | 14.33 | norathyriol-O-hexoside | XAN | 421.0768 | 0.58 | $[M-H]^-$ | $C_{19}H_{17}O_{11}$ | 259.02(100), 215.03(7), 421.08(2), 187.04(1) | 18.44 ±0.12 | M |
| 32 | 14.86 | quercetin-O-pentoside | FLA | 433.0767 | 0.42 | $[M-H]^-$ | $C_{20}H_{17}O_{11}$ | 300.03(100), 301.04(32), 271.03(37), 255.03(18), 151.00(6) | 35.42 ±0.33 | H |
| 33 | 14.94 | Kaempferol 3-O-glucoside (astragain) | FLA | 447.0932 | 2.20 | $[M-H]^-$ | $C_{21}H_{19}O_{11}$ | 447.09(100), 255.03(85), 284.03(81), 227.03(79), 285.04(30) | 108.83 ±0.74 | H |
| 34 | 15.34 | kaempferol-O- hexauronide | FLA | 461.0704 | 0.45 | $[M-H]^-$ | $C_{21}H_{17}O_{12}$ | 285.04(100), 113.03(14), 284.04(11), 229.05(11), 257.05(6) | TR[3] | |
| 35 | 15.36 | kaempferol-O-hexoside | FLA | 447.0930 | 1.86 | $[M-H]^-$ | $C_{21}H_{19}O_{11}$ | 447.09(100), 227.03(87), 255.03(83), 284.03(79), 285.04(44) | TR[3] | |
| 36 | 15.43 | Quercetin 3-O-rhamnoside (quercitrin) | FLA | 447.0931 | 2.13 | $[M-H]^-$ | $C_{21}H_{19}O_{11}$ | 300.03(100), 301.04(85), 271.03(44), 255.03(26), 151.00(16) | 1601.27 ±9.74 | H |
| 37 | 15.88 | maclurin-O-(benzoyl)-hexoside | BEN | 527.1189 | 0.94 | $[M-H]^-$ | $C_{26}H_{23}O_{12}$ | 151.00(100), 261.04(94), 405.08(92), 107.01(29) | 2271.27 ±1.77 | M |
| 38 | 15.91 | norathyriol-O-(benzoyl)-hexoside | XAN | 525.1041 | 2.50 | $[M-H]^-$ | $C_{26}H_{21}O_{12}$ | 259.02(100), 403.07(13), 215.03(8), 187.04(4) | 49.91 ±1.23 | M |
| 39 | 15.92 | myricetin | FLA | 317.0301 | 2.83 | $[M-H]^-$ | $C_{15}H_9O_8$ | 317.03(100), 151.00(53), 137.02(41), 178.99(35), 107.01(20), 193.01(2), 165.02 (3) | 350.47 ±0.71 | H |
| 40 | 16.37 | 5-hydroxy-2-isopropylchromone-7-O-glucoside | CHR | 427.1248 | 2.97 | $[M+HCOO]^-$ | $C_{19}H_{23}O_{11}$ | 219.07(100), 204.04(9), 203.03(9), 220.07(8), 381.12(3) | 175.27 ±1.92 | M |
| 41 | 17.86 | quercetin | FLA | 301.0353 | 3.32 | $[M-H]^-$ | $C_{10}H_9O_7$ | 301.04(100), 151.00(68), 178.99(29), 121.03(23), 107.01(20), 193.01 (1), 149.02 (3) | 410.91 ±2.99 | H |
| 42 | 18.18 | norathyriol-O-(benzoyl)-hexoside | XAN | 525.1036 | 1.57 | $[M-H]^-$ | $C_{26}H_{21}O_{12}$ | 259.02(100), 403.07(11), 215.03(8), 187.04(4) | 47.31 ±0.12 | M |
| 43 | 20.00 | 3,8'-biapigenin | FLD | 537.0826 | 1.44 | $[M-H]^-$ | $C_{30}H_{17}O_{10}$ | 151.00(100), 385.07(45), 443.04(19), 417.06(3) | 1697.85 ±3.91 | H |
| 44 | 20.70 | 3',8"-biapigenin (amentoflavone) | FLD | 537.0828 | 2.12 | $[M-H]^-$ | $C_{30}H_{17}O_{10}$ | 537.08(100), 375.05(98), 417.06(22), 443.04(9) | 50.24 ±0.33 | H |

[1] Classes of secondary metabolites: HBA – Hydroxybenzoic acids; BEN – Benzophenones; HCA – Hydroxycinnamic acids; FLO – Flavan-3-ols (catechins); XAN – Xanthones; FLA – Flavonols; CHR – Chromones; FLD – Flavone dimers.

[2] The quantity of the metabolites was calculated as: C – chlorogenic acid, M – mangiferin, and H – hyperoside.

[3] The metabolite was detected in traces.

royalquinic acid, respectively. Both metabolites **12** and **19** correspond to 5-O-p-coumaroylquinic acid while **8** and **11** were identified as 5-O-p-caffeoylquinic acid. There was a substantial difference in retention times of compounds **8**, **11**, **12**, and **19** which were 5.79, 8.19, 8.26, and 10.94 min., respectively that phenomenon corroborates with the recently reported data (Ncube et al. 2014). Thus, the metabolites **8**, **11**, **12**, and **19** were tentatively identified as 5-O-*trans*-p-caffeoylquinic acid, 5-O-*cis*-p-caffeoylquinic acid, 5-O-*trans*-p-coumaroylquinic acid, and 5-O-*cis*-p-coumaroylquinic acid, respectively. Furthermore, compound **17** showed a deprotonated molecule at *m/z* 367.1039. Its MS/MS spectrum showed a similar fragmentation pattern as compounds **3**,

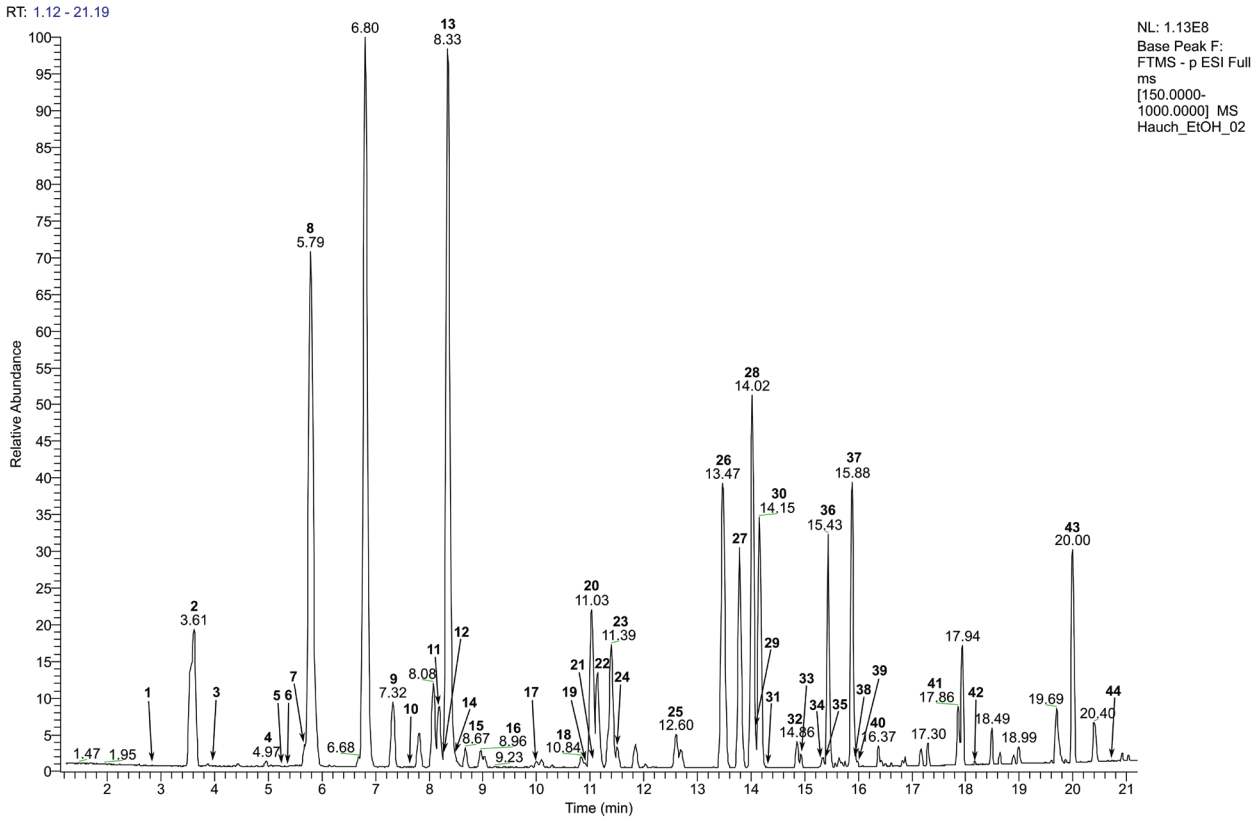


**Figure 2.** Chromatogram of the EtOH extract from the aerial parts of *Hypericum aucheri*.

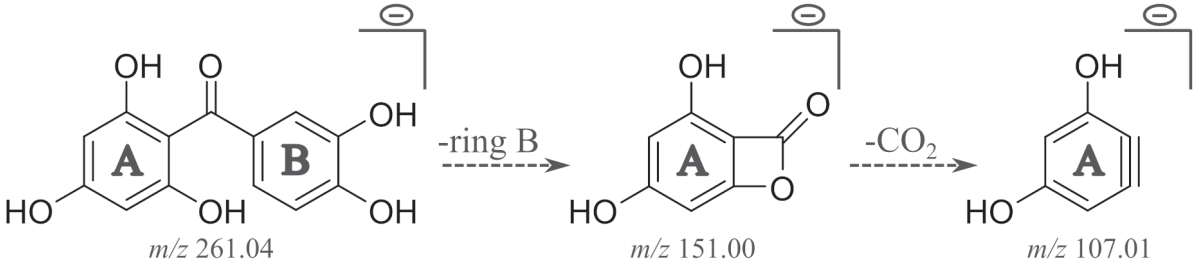


**Figure 3.** A postulated fragmentation pathway of maclurin.

**6**, **8**, **11**, **12**, and **19** but differed in the presence of a product ion at *m/z* 193.05 which was typical for ferulic acid derivatives. According to literature data, the metabolite **17** was tentatively identified as 5-O-feruloylquinic acid (Clifford et al. 2003; Masike et al. 2017). The deprotonated molecules $[M–H]^-$ of compounds **5** and **10** appeared at *m/z* 355.1037–355.1038. In the MS/MS spectra of both metabolites, the product ion at *m/z* 193.05 corresponded to 162 Da neutral loss which is typical for O-hexoses. In addition, the MS/MS spectrum of **5** showed fragments at *m/z* 134.04, 149.06, and 178.03 typical for ferulic acid (Sinosaki et al. 2020), while in those of **10** appeared product ions at *m/z* 217.05, 193.05, 175.04, and 160.02. Thus, metabolites **5** and **10** were tentatively identified as ferulic acid 4-O-hexoside and 1-O-feruloyl-β-glucose (Umehara et al. 2018), respectively. The total amount of hydroxycinnamic acid derivatives in *Hypericum aucheri* was 13.55 mg/g D.W. and was dominated by chlorogenic acid (>85% of the mixture). Excluding chlorogenic acid **8**, all other hydroxycinnamic acid derivatives were reported here for the first time for the title plant.

**Flavan-3-ols (catechins) and dimers**

The MS/MS spectra of the deprotonated molecules $[M–H]^-$ (*m/z* ranging from 289.0718 to 289.0720) of **7** and **14** showed product ions *m/z* 271.06, 179.03, 109.03, 151.02, 137.02, 125.02, 245.08, and 203.07. The loss of a water molecule (18 Da), catechol group (110 Da) and ring A and C (180 Da) yielded fragment ions at *m/z* 271.06, 179.03, and 109.03, respectively. The product ions at *m/z* 151.02 and 137.02 resulted from RDA reactions, while the main fragment from heterocyclic ring fusion had *m/z* 125.02. The loss of the $-CH_2$-CHOH- group from the benzofuran skeleton led to the formation of a fragment at *m/z* 245.08, which further decayed to an ion at *m/z* 203.07. Thus, metabolites **7** and **14** were tentatively identified as

catechin and epicatechin (Zeeb et al. 2000; Verardo et al. 2008; Yuzuak et al. 2018; Mutungi et al. 2021). The deprotonated molecule $[M–H]^-$ of **4** at *m/z* 305.0667 showed a similar fragmentation pattern, but in MS/MS produced a different fragment ion with *m/z* 287.06 corresponding to a water molecule loss (18 Da), suggesting that the compound is a hydroxy derivative of **7** and **14**. According to literature data **4** was tentatively identified as (+)-gallocatechin (Miketova et al. 2000). The deprotonated molecule $[M–H]^-$ of **9** appeared at *m/z* 577.1364. Its MS/MS spectrum showed characteristic product ions at *m/z* 125.02, 425.09, 407.08, 289.07, 245.08, 161.02 and 137.02. The fragment at *m/z* 425.09 was a product of the RDA reaction underwent subsequent water loss to give an ion with *m/z* 407.07. The product ion at *m/z* 289.07 corresponded to the catechin core derived from the quinone methide cleavage of the inter-flavonoid bond (Rue et al. 2018). Subsequently, the later ion underwent further fragmentations to produce fragments with *m/z* 245.08, 137.02, and 125.02 which corresponded to neutral loss of 44 Da, 152 Da, and 164 Da, respectively. Besides, the loss of 84 Da from the former ion produced a fragment at *m/z* 161. According to a recent data report compound **9** was tentatively identified as procyanidin B2 (Mutungi et al. 2021). The amount of the individual catechins was calculated as hyperoside. The total content of flavan-3-ols in the aerial parts of *H. aucheri* was estimated to be 1.24 mg/g D.W. dominated by epicatechin (59.50 %) and catechin (21.85%). Flavan-3-ols derivatives were reported here for the first time to occur in the title plant.

**Xanthones**

The deprotonated molecules $[M–H]^-$ of metabolites, **13**, **15, 16**, and **24** appeared at *m/z* 421.0776, 437.0722, 421.0771, and 405.0825, respectively. In the MS/MS spectrum the isobaric compounds **13** and **16** produced fragments at *m/z* 403.07, 259.03, 271.03, 301.04, and 331.05 while **24** showed product ions at *m/z* 387.07, 243.03, 255.03, 285.04, and 315.05. In addition, the MS/MS spectrum of the metabolite **15** showed product ions at *m/z* 419.06, 275.02, 287.02, 317.03, and 347.04. The above fragmentation pattern (Fig. 4) was characteristic of xanthone-C-glycosides resulted from water loss, C–C glycosidic bond split off, and $^{0,1}X^-$, $^{0,2}X^-$, $^{0,3}X^-$ cleavages of sugar moiety. The comparison of the MS/MS spectra with literature data, the compounds **13**, **16**, **15**, and **24** were tentatively identified as mangiferin, isomangiferin (Trevisan et al. 2016), pentahydroxyxanthone-C-hexoside and lancerin (Ling et al. 2013), respectively. Compounds **18** and **31** showed deprotonated molecules $[M–H]^-$ at *m/z* 421.0768–421.0775. Their MS/MS spectra revealed the presence of a base peak at *m/z* 259.02, corresponding to the loss of the hexose as a sugar moiety, which is typical of O-glycosides. Subsequently, the later ion gave a fragment at *m/z* 215.03 indicative of $CO_2$ loss followed by a cleavage of CO resulted in a fragment at *m/z* 187.04, which according to the literature data is typical of norathyriol (Heinrich et al. 2017; Islam et al. 2020). Thus, compounds **18** and **31**

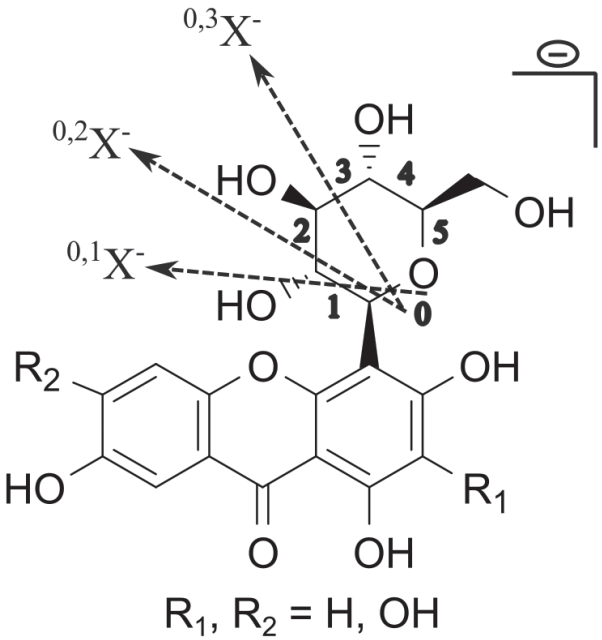


**Figure 4.** The fragmentation pattern of xanthone-C-glycosides.

were tentatively identified as norathyriol-O-hexoses. The deprotonated molecules of $[M–H]^-$ **21** and **25** appeared at *m/z* 421.0775, but their MS/MS spectra considerably differed in fragmentation pattern with product ions at *m/z* 259.02, 258.02 (base peak), 241.01, 229.01 and 213.02. The former fragment ion resulted from a loss of an O-glycosidic linked hexose (162 Da) and corresponded to the deprotonated form of the aglycone, which further lost a proton and producing the base peak (Wolfender et al. 1998). Subsequent fragmentation of the later ion showed loss of OH followed by CO cleavage giving product ions at *m/z* 241.01 and 213.02, respectively. Alternatively, the base peak (*m/z* 258.02) broke down to a fragment ion at *m/z* 229.01 which was indicative of CHO loss. Thus, compounds **21** and **25** were tentatively identified as 1,3,5,6-tetrahydroxyxanthone-O-hexosides (Tusevski et al. 2013). The deprotonated molecules $[M–H]^-$ of compounds **38** and **42** appeared at *m/z* 525.1041 and 525.1036, respectively. The MS/MS spectra showed product ion at *m/z* 403.06 which was an indication of a benzoic acid split off from the precursor (neutral loss of 122 Da), while the fragment at *m/z* 259.02 resulted from the loss of a benzoylated hexose $[M–H–C_{13}H_{14}O_6]^-$ and corresponds to the aglycon. Subsequently, the later product ion gave characteristic fragments at *m/z* 215.03 and 187.04 indicative of $CO_2$ and $C_2O_3$ losses, respectively that was typical for norathyriol (Heinrich et al. 2017; Islam et al. 2020). Thus, compounds **38** and **42** were tentatively identified as norathyriol-O-(benzoyl)-hexosides. The total amount of xanthones in *H. aucheri* was found to be 10.34 mg/g D.W. Mangifern **13** was the main constituent (87.77%) of this mixture. Except for norathyriol, mangiferin, and isomangiferin, all other xanthones were reported here for the first time to occur in the title plant while compounds **15**, **38,** and **42** were tentatively established as new natural products.

**Flavonol aglycones and their glycosides**

The deprotonated molecules $[M–H]^-$ of thirteen flavonol-O-glycosides, namely myricetin-O-hexosides **20** and **23** (*m/z* 479.0827 and 479.0828), myricetin-O-hexauronides **22** and **29** (*m/z* 493.0621 and 493.0636), myricetin-O-deoxyhexoside **26** (*m/z* 463.0881), quercetin-O-hexosides **27** and **30** (*m/z* 463.0880 and 463.0881), quercetin-O-hexauronide **28** (*m/z* 477.0670), quercetin-O-pentoside **32** (*m/z* 433.0767), quercetin-O-deoxyhexoside **36** (*m/z* 447.0931), kaempferol-O-hexosides **33** and **35** (*m/z* 447.0932 and 447.0930), and kaempferol-O-hexauronide **34** (*m/z* 461.0704), were detected in the full scan MS spectrum. In the MS/MS spectra, the corresponding precursor ions and the neutral losses of 134 Da (for **32**), 147 Da (for **26**, **36**), 163 Da (for **20**, **23**, **27**, **30**, **33**, and **35**), and 176 Da (for **22**, **28**, **29**, and **34**) indicated the presence of pentose, deoxyhexose, hexose, and hexauronic acid as sugar moieties, respectively. Moreover, characteristic fragment ions at *m/z* 317.03 (for **20**, **22**, **23**, **26**, and **29**), 301.04 (for **27**, **28**, **30**, **32** and **36**), and 285.04 (for **33**, **34**, and **35**) corresponded to the deprotonated molecules of myricetin, quercetin, and kaempferol, while fragment ions at *m/z* 316.02, 300.03, and 284.03 (all being the base peaks), respectively, were derived from homolytic cleavage of the glycosidic bond (Cuyckens and Claeys 2004). Furthermore, the MS/MS spectra of the deprotonated molecules $[M–H]^-$ of **39** (*m/z* 317.0301) and **41** (*m/z* 301.0353) exhibited similar product ions at *m/z* 193.01, 151.00, 107.01, and 178.99, resulting from $[M–H–\text{ring B}]^-$ loss, $^{1,3}A^-$, $^{0,4}A^-$ retro Diels-Alder (RDA) fragmentation pathways (Fig. 5), and $^{1,2}A^-$ retrocyclization, respectively. Furthermore, characteristic fragments at *m/z* 137.02 and 165.02 for **39** and at *m/z* 121.03 and 149.02 for **41** were observed due to $^{1,2}B^-$ retrocyclization and $^{1,3}B^-$ cleavage. Following the nomenclature applied by Fabre et al. (Fabre et al. 2001), the compounds were identified as myricetin **39** and quercetin **41**. The flavonols represent the major group of polar phenolics in the aerial parts of *H. aucheri* estimating a total of 16.54 mg/g D.W. Quercetin and its glycosides were dominant in the mixture estimating 57.16% of it followed by myricetin and its glycosides (42.18%).

**Chromones**

In the full MS scans, compound **40** appeared as formate adduct $[M+HCOO]^-$ at *m/z* 427.1248. The MS/MS spectrum showed a product ion at *m/z* 381.12 corresponded to the deprotonated molecule of **40** and a base peak ion at *m/z* 219.06 that indicated a loss of a hexose unit. Thus, compound **40** was tentatively identified as a 5-hydroxy-2-isopropylchromone 7-O-glucoside (An et al. 2009). It was reported here for the first time to occur in the studied plant. The amount of **40**, expressed as hyperoside, was found to be 175.27 µg/g D.W.

**Biflavones**

In the full MS spectrum, the deprotonated molecules $[M–H]^-$ of the isobaric compounds **43** and **44** appeared at *m/z* 537.0826 537.0828, respectively. Their MS/MS spectra showed similar product ions at *m/z* 443.04 and 417.06, resulting from $[M–H–C_6H_6O]^-$ and $[M–H–C_9H_6O_3]^-$ losses and characteristic fragments $[M–H–C_7H_4O_4]^-$ at *m/z* 385.07 for **43** and $[M–H–C_6H_6O]^-$ at *m/z* 375.05 for **44**. Furthermore, the RDA reaction of **44** led to split off a ketene derivative (*m/z* 162) of 4-hydroxycinnamic acid, whereas the compound **43** showed a cleavage of a phloroglucinol derivative (*m/z* 151). According to the literature data (Michler et al. 2011; Zhang et al. 2012) the compounds **43** and **44** were identified as 3,8'-biapigenin and 3',8"-biapigenin (amentoflavone), respectively. The amount of the former compound **43** was found to be 3.45% (1697.85 µg/g D.W.) of the total phenolic mixture while later **44** was found in very small quantities (50.24 µg/g D.W.). The amentoflavone **44** was reported here for the first time to occur in *H. aucheri*.

## Conclusions

A novel UHPLC-HRMS method was developed and applied for the identification and quantification of the polar phenolic compounds detected in the EtOH extract from the aerial parts of *H. aucheri*. The method was validated for specificity, the limit of detection and quantitation limit, linearity, accuracy, and precision. The external standards, chlorogenic acid, mangiferin, and hyperoside

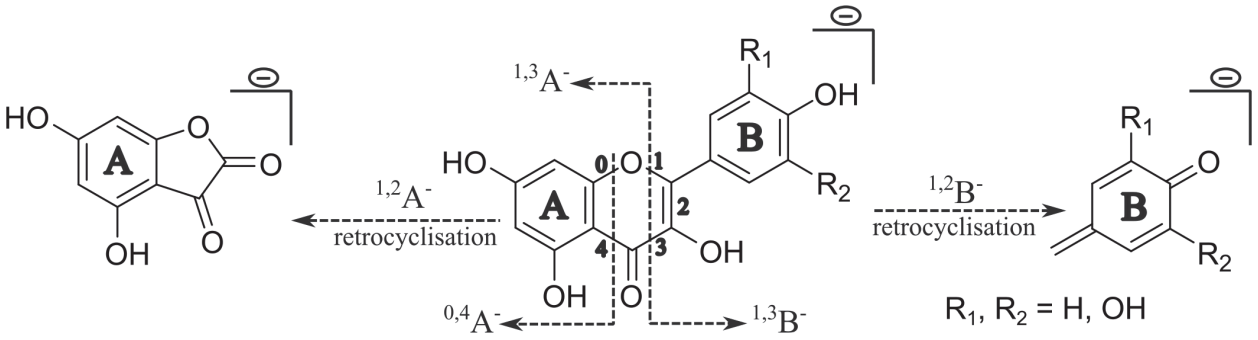


**Figure 5.** A postulated fragmentation pathway of flavanols.

were selected in this analysis. A total of 44 compounds, belonging to eight classes of phenolic secondary metabolites, were detected and quantified in the aerial parts of *H. aucheri*. Pentahydroxyxanthone-C-glycoside **15**, maclurin-O-(benzoyl)-hexoside **37**, and norathyriol-O-(benzoyl)-hexosides **38** and **42** were suggested to be new natural compounds, while maclurin-O-hexoside **2** was reported for the first time for *Hypericum* genus. Additionally, more than 22 secondary metabolites, including benzophenones, hydroxycinnamic acid derivatives, catechins, and a chromone, were identified for the first time in *H. aucheri*. The amounts of the detected metabolites were calculated relative to external standards. The dominant polar phenolic constituents were chlorogenic acid (11.55 mg/g D.W.) and mangiferin (9.13 mg/g D.W.). The developed UHPLC-HRMS method can be used to identify and quantify polar phenolic compounds in the aerial parts of other *Hypericum* species.

## Acknowledgments

This study was supported by the European Union-NextGenerationEU through the National Recovery and Resilience Plan of the Republic of Bulgaria grant number № BG-RRP-2.004-0004-C01.

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