DOI: 10.1002/cbdv.201800565

FULL PAPER

CHEMISTRY & BIODIVERSITY

Phytochemical Analysis and Total Antioxidant Capacity of Rhizome, Above-Ground Vegetative Parts and Flower of Three *Iris* Species

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This study was aimed at investigating the phytochemical composition and antioxidant capacity of rhizomes, above-ground vegetative parts and flowers of three *Iris* species: *Iris humilis* Georgi, *Iris pumila* L. and *Iris variegata* L. UHPLC-Orbitrap MS analysis was used for determination of phytochemical profile. Total pigments, phenolics, flavonoids, soluble sugars and starch content as well as ABTS antioxidant capacity were also determined. In total, 52 phenolics compounds were identified with 9 compounds (derivatives of iriflophenone, apigenin *C*-glycosides, luteolin *O*-glycoside, isoflavones derivatives of iristectorigenin, dichotomitin, nigracin and irilone) never reported before in *Iris* spp. Differences in phenolic composition profile, pigments, soluble sugar, starch, total phenolics and flavonoids content and total antioxidant capacity were found among *Iris* species and different part of plants. Significant correlation between total phenolic content and antioxidant capacity was determined. The obtained results are comparable with those obtained for medical plants. These findings could be useful for fingerprinting characterization of *Iris* species and estimation of possible use in pharmaceutical industries.

Keywords: *Iris humilis*, *Iris pumila*, *Iris variegate*, phenolics, LC/MS, phytochemistry.

Introduction

Iridaceae represents widely distributed plant family (especially in temperate and tropical climatic zones) that including 92 genera and about 1800 species.[1,2] Among them, *Iris* is one of the most important genera of flowering plants with significant contribution to wild habitats of Eurasia and North America.[2,3] *Iris* species are rich in different secondary metabolites content.[2,3] Most phytochemical analyzes among *Iris* genera were performed on *I. germanica* (German iris) since it is commonly grown as ornamental plant in gardens and parks.[4 – 8] Information on phytochemical composition (especially flavonoids/isoflavones profiles) of *I. pallida*,[7,8] *I. albicans*,[8] *I. kashmiriana*[9] and *I. lutescens*[10] are also available. According to literature,[3] 122 different compounds are detected in eleven *Iris* species. Most of them belong to flavonoids, simple phenolics, steroids and terpenoids. It is well-known that phenolic compounds are among the most widespread class of secondary metabolites in plants that are characterized by antioxidant and antimicrobial properties. Different secondary metabolites can cause a healing effect for some diseases in human, including cancer. In the case of some *Iris* species, pharmacological activity has been confirmed several times[2,3,11 – 13] as well as antimicrobial activity.[14,15] *I. pallida* and *I. germanica* are commercially grown in Italy, Morocco and France for oil production from roots which has been used as precious and one of the most expensive component in perfume industry.[3,16]

I. humilis, *I. pumila* L. and *I. variegata* L. are native to Eurasia including Serbia. *I. humilis* subsp. *arenaria* (Waldst. & Kit.) Á.Löve & D.Löve (hereinafter *I. humilis*) is a Pontic-Pannonian endangered and protected species

(in Czech Republic, Slovakia, Hungary and Serbia) occurring in southeastern and southern part of Central Europe. This is a pioneer species of sandstone (*Festucion vaginatae*) and steppe (*Festucion rupicolae*) habitats, but in the spontaneous extinction. *I. variegata* inhabits areas of central and southeastern Europe. It grows on grassy and open forest habitats. *I. pumila* is a rhizomatous perennial clonal species widely distributed in the lowlands of Central and Southeast Europe. In Serbia, it is abundant in the dune system of the special nature reserve – Deliblato Sands.[17] However, very limited information is available on their phytochemicals composition, iron content in rhizomes of *I. variegata* as important component for perfume industry[18,19] and total anthocyanins content of *I. pumila* leaves.[20] Literature review revealed that there is no available information about chemical composition of rhizomes, green parts (stem and leaves) and flowers of *I. humilis*.

Further, phenolics are well-known as potential tool for chemotaxonomic characterization for different plant species[21–26] or materials such as pollen[27] and honey.[28] Knowing that xanthone, isoflavone and flavonoid derivatives are almost exclusively present in Iridaceae family plants[29] and antioxidant properties of polyphenols, the aim of this work was to characterize the phytochemical composition and antioxidant properties of rhizomes, green parts and flowers of three mentioned *Iris* species. The obtained results could be valuable for possible use of phenolic profiles as 'botanical fingerprint' of *Iris* species and estimation of their possible use in pharmaceutical industries.

Results and Discussion

Phytochemical Profile

UHPLC-Orbitrap MS characterization of three *Iris* extracts in a negative ionization mode resulted in the detection of 52 compounds in total. The identified compounds represented four structurally distinct groups: 1) xanthone and their derivatives (12 compounds); 2) flavonoid *C*-glycosides (8 compounds); 3) flavonoid *O*-glycosides (11 compounds); and 4) isoflavones and their derivatives (21 compounds). Chemical structures of phytochemicals found in three investigated *Iris* species are shown in *Figure 1*.

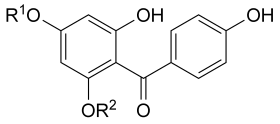
Among all identified compounds, six were confirmed using standards, while the others were identified by exact mass search of their deprotonated molecule $[M - H]^-$, MS^2, MS^3 and MS^4 fragmentation behavior, as well as by comparison with the available literature. The peak numbers, compound names, molecular formulas, calculated and exact masses ($[M - H]^-$, *m/z*), mean mass accuracy errors (mDa), as well as presence of selected compound in various parts of three *Iris* species are summarized in *Table 1*, while the retention times (t_R, min) and major MS^2, MS^3 and MS^4 fragment ions are summarized in *Table 2*.

Xanthones

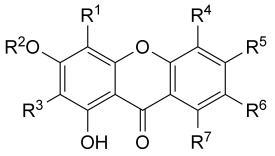
Xanthones, commonly present in *Iris* species,[13] in our study were found as free and in the form of glycosides.

Xanthone derivative, iriflophenone (compound **10**), which in its narrow structure is actually benzophenone, and four of their derivatives were identified in several of tested samples (*Table 1*). Two isomeric iriflophenone derivatives, **1** (3.90 min) and **3** (4.92 min), with identical molecular ion ($[M-H]^-$ at 407 *m/z*), but showing slightly different MS fragmentation patterns, were identified as iriflophenone 4-*O*-hexoside and iriflophenone 2-*O*-hexoside, respectively. Both compounds generated MS^2 base peak at 245 *m/z* (loss of hexoside; 162 Da) corresponding to deprotonated iriflophenone. By studying the MS^3 fragmentation patterns of these two derivatives, the existence of a 161 *m/z* fragment was found to be characteristic for iriflophenone 2-*O*-hexoside.[13] In addition, iriflophenone 4-*O*-(6"-acetyl)hexoside (compound **6**) and 4-*O*-methyliriflophenone (compound **11**) were also identified. Compound **6** at 5.93 min and 449 *m/z* generated MS^2 base peak at 245 *m/z* and MS^2 secondary peak at 389 *m/z* (corresponding to loss of acetic acid – 60 Da). The present study provides the first report of tentative identification of iriflophenone 4-*O*-(6"-acetyl)hexoside in some herbs belonging to *Iris* species. Compound **11** was previously reported in *I. germanica* and *I. pallida* extracts.[7]

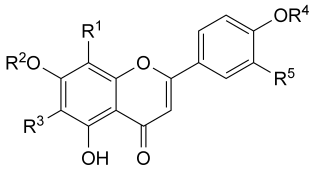
As for other xanthones, three compounds (**2**, **5** and **9**) at the same $[M-H]^-$ (421 *m/z*) were identified as mangiferin, isomangiferin and nigricanside, respectively. Tentative identification of these compounds was based on chromatographic and MS data previously reported.[30] Confirmation of compound **9** was based on existence of a 383 *m/z* fragment in MS^2 spectrum, which were absent in the case of the other two above-mentioned isomers.[30] Compound **5** were the only compound found in all samples (all three *Iris* species; in rhizome, above-ground vegetative parts and flower). Compounds **4** and **8**, with the same accurate mass (435 *m/z*) and very similar fragmentation patterns, were marked as 7-*O*-methylmangiferin



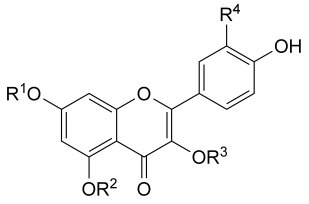
Compound	R^1	R^2
1	Hex	H
3	H	Hex
6	Ac(1→6)Hex	H
10	H	H
11	Me	H



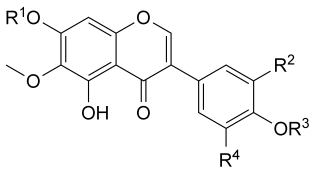
Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7
2	H	H	Hex	H	OH	OH	H
4	H	H	Hex	H	OH	MeO	H
5	Hex	H	H	H	OH	OH	H
7	H	H	Pent(1→6)Hex	H	OH	OH	H
8	Hex	H	H	H	OH	MeO	H
9	H	H	Hex	OH	H	H	OH
12	H	Me	H	OH	H	H	OH



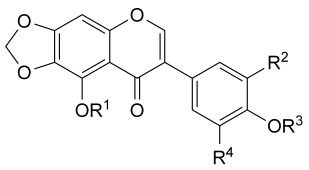
Compound	R^1	R^2	R^3	R^4	R^5
13	Hex	H	H	H	OH
14	H	H	Hex	H	OH
15	Hex(1→2)Hex	H	H	H	H
16	Hex(1→2)Hex	H	H	H	H
17	Hex	H	H	H	H
18	H	H	Hex	H	H
19	Hex	H	H	Me	H
20	H	H	Hex	Me	H
31	H	*p*-Cou(1→2)Rham	H	H	OH



Compound	R^1	R^2	R^3	R^4
21 and **23**	H	H	Hex	OH
22	H	H	Rham(1→2)Hex	MeO
24	Rham(1→6)Hex	H	H	H
25	Me	Hex	H	MeO
26	H	H	Rham(1→6)Hex	OH
27 and **29**	H	H	Hex	H
28 and **30**	H	H	Hex	MeO



Compound	R^1	R^2	R^3	R^4
32	Hex	H	H	H
33	Hex	MeO	H	H
34	HexA	OH	Me	H
35	Hex	OH	Me	H
37	Hex	OH	H	H
38	Hex	MeO	Me	OH
40	Me	H	Hex(1→6)Hex	H
44	Hex	H	Me	H
45	H	H	H	H
48	H	OH	H	H
49	H	OH	H	MeO
52	H	H	H	H



Compound	R^1	R^2	R^3	R^4
36	H	H	Hex(1→6)Hex	H
39	Hex	MeO	Me	O-Hex(1→6)Hex
41	H	MeO	HMG(1→6)Hex	H
42	H	H	Hex	MeO
43	H	H	Hex	H
46	H	MeO	Me	O-Hex
47	H	H	HMG(1→6)Hex	H
50	H	H	H	H
51	H	H	H	MeO

Figure 1. Structures of phytochemicals found in rhizomes, green parts (stem and leaves) and flowers of three *Iris* sp. (*I. humilis*, *I. pumila* and *I. variegata*); Hex – hexosyl; Ac – acetyl; Pent – pentosyl; Rham – rhamnosyl; *p*-Cou – *p*-coumaroyl; HexA – hexuronyl; HMG – 3-hydroxy-3-methylglutaryl.

and 7-*O*-methylisomangiferin (*Table 2*).[30] Compound **7** at 6.23 min and 567 *m/z* was tentatively marked as polygalaxanthone III, according to available literature about chemical constituents in Kai-Xin-San herb formula.[31] The last one from the xanthones group, bellidifolin (273 *m/z*; compound **11**), previously isolated from rhizomes of *I. nigricans*,[32] was found in the current study in *I. pumila* rhizome and above-ground vegetative parts (*Table 1*). It produced MS^2 base peak at 258 *m/z* (corresponding to loss of methyl group) and MS^3 base peak at 230 *m/z* (formed by further loss of CO group).

Table 1. High resolution MS data of phytochemicals found in *Iris* spp.[a]

Peak No.	Compound name	Molecular formula $[M-H]^-$	Calculated mass $[M-H]^-$	Exact mass $[M-H]^-$	Δ mDa	*Iris humilis* R	*Iris humilis* AGP	*Iris humilis* F	*Iris pumila* R	*Iris pumila* AGP	*Iris pumila* F	*Iris variegata* R	*Iris variegata* AGP	*Iris variegata* F
Xanthones														
1	Iriflophenone 4-*O*-hexoside	$C_{19}H_{19}O_{10}^-$	407.09837	407.09503	3.34	−	−	−	+	−	−	+	+	+
2	Mangiferin	$C_{19}H_{17}O_{11}^-$	421.07763	421.07440	3.23	−	−	+	+	+	+	+	+	+
3	Iriflophenone 2-*O*-hexoside	$C_{19}H_{19}O_{10}^-$	407.09837	407.09564	2.73	−	−	−	+	−	−	+	+	+
4	7-*O*-Methylmangiferin	$C_{20}H_{19}O_{11}^-$	435.09329	435.08981	3.48	−	−	−	+	−	−	+	−	−
5	Isomangiferin	$C_{19}H_{17}O_{11}^-$	421.07763	421.07425	3.38	+	+	+	+	+	+	+	+	+
6	Iriflophenone 4-*O*-(6″-acetyl)hexoside	$C_{21}H_{21}O_{11}^-$	449.10893	449.10536	3.57	+	−	−	−	−	−	−	−	−
7	Polygalaxanthone III	$C_{25}H_{27}O_{15}^-$	567.13554	567.13086	4.68	+	−	−	−	−	−	−	−	−
8	7-*O*-Methylisomangiferin	$C_{20}H_{19}O_{11}^-$	435.09329	435.09012	3.17	+	−	−	+	+	+	+	−	−
9	Nigricanside	$C_{19}H_{17}O_{11}^-$	421.07763	421.07401	3.62	−	−	−	−	−	−	+	−	−
10	Iriflophenone	$C_{13}H_9O_5^-$	245.04555	245.04370	1.85	+	−	−	+	+	+	+	+	+
11	4-*O*-Methyliriflophenone	$C_{14}H_{11}O_5^-$	259.06120	259.05939	1.81	+	−	−	+	+	−	+	+	+
12	Bellidifolin	$C_{14}H_9O_6^-$	273.04046	273.03815	2.31	−	−	−	+	+	−	−	−	−
Flavonoid *C*-glycosides														
13	Luteolin 8-*C*-hexoside	$C_{21}H_{19}O_{11}^-$	447.09329	447.08975	3.54	−	−	−	−	−	+	−	−	−
14	Luteolin 6-*C*-glucoside	$C_{21}H_{19}O_{11}^-$	447.09329	447.08987	3.42	−	−	−	−	+	+	−	+	+
15	Apigenin 8-*C*-(2″-hexosyl)hexoside	$C_{27}H_{29}O_{15}^-$	593.15119	593.14642	4.77	−	−	−	−	−	+	−	−	−
16	Apigenin 8-*C*-(2″-pentosyl)hexoside	$C_{26}H_{27}O_{14}^-$	563.14063	563.13611	4.52	−	−	−	−	−	+	−	−	−
17	Apigenin 8-*C*-glucoside	$C_{21}H_{19}O_{10}^-$	431.09837	431.09515	3.22	−	−	−	−	+	+	−	+	−
18	Apigenin 6-*C*-hexoside	$C_{21}H_{19}O_{10}^-$	431.09837	431.09500	3.37	−	−	−	−	+	+	−	+	−
19	4′-*O*-Methylapigenin 8-*C*-hexoside	$C_{22}H_{21}O_{10}^-$	445.11402	445.11096	3.06	−	−	−	−	+	−	−	+	+
20	4′-*O*-Methylapigenin 6-*C*-hexoside	$C_{22}H_{21}O_{10}^-$	445.11402	445.11041	3.61	−	−	−	−	+	−	−	+	+
Flavonoid *O*-glycosides														
21	Quercetin 3-*O*-galactoside	$C_{21}H_{19}O_{12}^-$	463.08820	463.08426	3.94	−	−	+	−	−	−	−	−	−
22	Isorhamnetin 3-*O*-(2″-rhamnosyl)hexoside	$C_{28}H_{31}O_{16}^-$	623.16176	623.15715	4.61	−	−	+	−	−	−	−	−	−
23	Quercetin 3-*O*-glucoside	$C_{21}H_{19}O_{12}^-$	463.08820	463.08423	3.97	−	−	+	−	−	−	−	−	−
24	Kaempferol 7-*O*-(6″-rhamnosyl)hexoside	$C_{27}H_{29}O_{15}^-$	593.15119	593.14636	4.83	−	−	+	−	−	+	−	−	+
25	Irisdichotin B	$C_{23}H_{25}O_{12}^-$	493.13515	493.13168	3.47	+	−	−	+	−	−	+	−	−
26	Isorhamnetin 3-*O*-(6″-rhamnosyl)hexoside	$C_{28}H_{31}O_{16}^-$	623.16176	623.15764	4.12	−	−	+	−	−	−	−	−	−
27	Kaempferol 3-*O*-galactoside	$C_{21}H_{19}O_{11}^-$	447.09329	447.08942	3.87	−	+	+	−	−	−	−	−	−
28	Isorhamnetin 3-*O*-galactoside	$C_{22}H_{21}O_{12}^-$	477.10385	477.10022	3.63	−	−	+	−	−	−	−	−	−
29	Kaempferol 3-*O*-glucoside	$C_{21}H_{19}O_{11}^-$	447.09329	447.08945	3.84	−	+	+	−	−	−	−	−	−
30	Isorhamnetin 3-*O*-glucoside	$C_{22}H_{21}O_{12}^-$	477.10385	477.09991	3.94	−	−	+	−	−	−	−	−	−
31	Luteolin 7-*O*-(2″-*p*-coumaroyl)rhamnoside	$C_{30}H_{25}O_{12}^-$	577.13515	577.13068	4.47	−	−	−	+	+	−	−	−	−
Isoflavones and derivatives														
32	Tectoridin	$C_{22}H_{21}O_{11}^-$	461.10893	461.10478	4.15	−	−	−	+	−	−	+	−	−
33	Iristectorin B	$C_{23}H_{23}O_{12}^-$	491.11950	491.11554	3.96	−	−	−	+	+	−	+	−	−
34	Iristectorigenin A 7-*O*-hexuronide	$C_{23}H_{21}O_{13}^-$	505.09876	505.09464	4.12	−	+	−	−	−	−	−	+	−
35	Iristectorin A	$C_{23}H_{23}O_{12}^-$	491.11950	491.11588	3.62	−	−	−	+	+	−	+	−	−
36	Irilone 4′-*O*-(6″-hexosyl)hexoside	$C_{28}H_{29}O_{16}^-$	621.14611	621.14203	4.08	+	−	−	−	−	−	−	−	−
37	3′-Hydroxyltectoridin	$C_{22}H_{21}O_{12}^-$	477.10385	477.10043	3.42	−	−	−	−	−	−	+	−	−
38	Iridin	$C_{24}H_{25}O_{13}^-$	521.13006	521.12610	3.96	+	−	−	+	−	−	+	−	−
39	Dichotomitin 3′-*O*-(6″-hexosyl)hexoside	$C_{30}H_{33}O_{18}^-$	681.16724	681.16233	4.91	+	−	−	−	−	−	−	−	−
40	7-*O*-Methyltectorigenin 4′-*O*-(6″-hexosyl)hexoside	$C_{29}H_{33}O_{16}^-$	637.17741	637.17310	4.31	+	−	−	+	−	−	+	−	−

Table 1. (cont.)

Peak No.	Compound name	Molecular formula $[M-H]^-$	Calculated mass $[M-H]^-$	Exact mass $[M-H]^-$	Δ mDa	*Iris humilis*			*Iris pumila*			*Iris variegata*		
						R	AGP	F	R	AGP	F	R	AGP	F
Xanthones														
41	Nigracin 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside	$C_{30}H_{32}O_{16}^-$	647.16176	647.15692	4.84	+	–	–	–	–	–	–	–	–
42	Irifloside	$C_{23}H_{21}O_{12}^-$	489.10385	489.09981	4.04	+	–	–	+	–	–	+	–	–
43	Irilone 4′-*O*-hexoside	$C_{22}H_{19}O_{11}^-$	459.09328	459.08973	3.55	+	–	–	+	–	–	+	–	–
44	Irisolidone 7-*O*-hexoside	$C_{23}H_{23}O_{11}^-$	475.12458	475.12178	2.80	+	–	–	+	–	+	+	+	–
45	Tectorigenin	$C_{16}H_{11}O_6^-$	299.05611	299.05347	2.64	+	+	–	+	+	–	+	–	–
46	Dichotomitin 3′-*O*-hexoside	$C_{24}H_{23}O_{13}^-$	519.11441	519.11078	3.63	+	–	–	–	–	–	–	–	–
47	Irilone 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside	$C_{28}H_{27}O_{15}^-$	603.13554	603.13055	4.99	–	–	–	–	+	–	–	–	–
48	Iristectorigenin A	$C_{17}H_{13}O_7^-$	329.06668	329.06372	2.96	–	+	–	+	+	–	+	+	–
49	Irigenin	$C_{18}H_{15}O_8^-$	359.07724	359.07422	3.02	+	–	–	+	+	–	+	–	–
50	Irilone	$C_{16}H_9O_6^-$	297.04046	297.03809	2.37	–	–	–	+	–	–	+	–	–
51	Iriflogenin	$C_{17}H_{11}O_7^-$	327.05103	327.04840	2.63	–	–	–	+	–	–	+	–	–
52	Irisolidone	$C_{17}H_{13}O_6^-$	313.07176	313.06857	3.19	–	–	–	+	–	–	+	–	–

[a] Peak No. – peak numbers (corresponding to *Figure 1*); mDa – mean mass accuracy; R – rhizome; AGP – above-ground vegetative parts; F - flower; + stands for detected and – stands for not detected compound.

Flavonoid C-Glycosides

From the flavonoid *C*-glycoside group, flavone derivatives (apigenin and luteolin) were found in our samples and their identification was largely based on the evaluated MS fragments and previously reported spectroscopic data about phytochemicals found in various *Iris* species.[33,34] Presence of compounds **14** and **17** (luteolin 6-*C*-glucoside and apigenin 8-*C*-glucoside) were confirmed using available standards. Specific fragmentation pattern of this two compounds, as well as their isomers, compounds **13** and **18** (luteolin 8-*C*-glucoside and apigenin 6-*C*-glucoside) were found in literature.[35] Compounds **15** (6.15 min; 593 *m/z*) and **16** (6.24 min; 593 *m/z*) with similar fragmentation pathway were identified only in *I. pumila* flower, as apigenin 8-*C*-(2″-hexosyl)hexoside and apigenin 8-*C*-(2″-pentosyl)hexoside, respectively. A search of literature did not find that such compounds were isolated from *Iris* species before, but their fragmentation is well-known and described in the literature.[36] Peaks **19** and **20**, with the same accurate mass but different ions in MS spectrum, were tentatively identified as 4′-*O*-methylapigenin 8-*C*-hexoside and 4′-*O*-methylapigenin 6-*C*-hexoside, respectively. These compounds were already isolated and identified in rhizomes of *I. pseudopumila*.[34]

Flavonoid O-Glycosides

Among eleven flavonoid *O*-glycosides, four of them were identified using available standards (quercetin 3-*O*-galactoside (**21**), quercetin 3-*O*-glucoside (**23**), kaempferol 3-*O*-glucoside (**29**) and isorhamnetin 3-*O*-glucoside (**30**)). Kaempferol 3-*O*-galactoside (**27**) was already described in *I. pseudopumila* rhizome.[34] Isorhamnetin 3-*O*-galactoside (**28**) was found only in *I. humilis* ssp. *arenaria* flower in the present study. Derivatives with the same molecular mass showing very similar fragmentation pathways were marked as galactose and glucose isomers, although it is known that galactoside has a shorter retention time.[37] By studying MS fragmentation of two isorhamnetin derivatives (compounds **22** and **26**) at 623 *m/z*, it can be concluded from the results of the present study that these two derivatives differ by interglycosidic linkage between sugars,[38] and they were marked as isorhamnetin 3-*O*-(2″-rhamnosyl)hexoside and isorhamnetin 3-*O*-(6″-rhamnosyl)hexoside, respectively. Compound **22** was already characterized in *I. hookeriana* rhizome.[39] Compound **24** at 6.77 min and 593 *m/z* gave MS^2 base peak at 285 *m/z* and MS^3 spectrum

Table 2. Negative ion mode MS^4 fragmentation data of phytochemicals found in *Iris* spp.

Peak No.[a]	Compound name	t_R [min]	Parent ion [*m/z*]	MS^2 Fragments [*m/z*] (% base peak)	MS^3 Fragments [*m/z*] (% base peak)	MS^4 Fragments [*m/z*] (% base peak)
Xanthones						
1	Iriflophenone 4-*O*-hexoside	3.90	407	359 (10), 287 (15), 245 (100)	201 (30), 157 (5), 151 (100), 125 (10), 107 (15)	107 (100), 83 (20), 65 (5)
2	Mangiferin	4.21	421	403 (20), 331 (75), 301 (100)	273 (60), 258 (100), 229 (5), 191 (5)	258 (50), 229 (50), 214 (100), 108 (30)
3	Iriflophenone 2-*O*-hexoside	4.92	407	245 (100)	201 (25), 177 (5), 161 (5), 151 (100), 125 (15)	107 (100), 83 (5)
4	7-*O*-Methylmangiferin	5.30	435	417 (10), 399 (10), 357 (10), 345 (20), 315 (100)	300 (20), 272 (100)	–[d]
5	Isomangiferin	5.50	421	403 (20), 331 (70), 301 (100)	273 (60), 258 (100), 229 (5), 191 (10), 137 (10)	241 (20), 230 (100), 203 (80), 188 (40), 158 (10)
6	Iriflophenone 4-*O*-(6″-acetyl)hexoside[b]	5.93	449	389 (10), 287 (5), 245 (100)	201 (50), 177 (5), 151 (100), 125 (15), 107 (10)	–
7	Polygalaxanthone III[b]	6.23	567	486 (10), 399 (10), 345 (40), 315 (100), 272 (20)	300 (40), 272 (100)	–
8	7-*O*-Methylisomangiferin	6.30	435	417 (10), 345 (30), 315 (100), 300 (5)	300 (25), 272 (100)	272 (20), 255 (10), 243 (100), 227 (40), 199 (20)
9	Nigricanside	6.59	421	403 (10), 383 (5), 331 (90), 301 (100), 281 (10)	284 (10), 273 (100), 258 (70), 230 (20), 165 (20)	–
10	Iriflophenone	6.83	245	201 (10), 171 (10), 175 (5), 151 (100), 125 (5)	107 (100), 83 (5), 65 (10)	65 (100)
11	4-*O*-Methyliriflophenone	8.51	259	222 (15), 191 (5), 165 (100)	150 (40), 121 (100), 97 (15), 91 (5), 65 (15)	–
12	Bellidifolin	10.34	273	259 (15), 258 (100)	258 (10), 230 (100), 229 (70), 213 (10), 202 (20)	–
Flavonoid *C*-glycosides						
13	Luteolin 8-*C*-hexoside	4.98	447	429 (15), 401 (10), 371 (10), 357 (100), 327 (90)	–	–
14	Luteolin 6-*C*-glucoside[c]	5.99	447	429 (20), 411 (5), 357 (60), 327 (100)	299 (100), 284 (10)	281 (40), 271 (50), 255 (100), 243 (40), 227 (50)
15	Apigenin 8-*C*-(2″-hexosyl)hexoside[b]	6.15	593	413 (100), 341 (10), 311 (5), 307 (10), 293 (30)	–	–
16	Apigenin 8-*C*-(2″-pentosyl)hexoside[b]	6.24	563	515 (5), 433 (5), 413 (100), 355 (10), 293 (35)	293 (100)	264 (20), 251 (20), 237 (20), 219 (25), 173 (100)
17	Apigenin 8-*C*-glucoside[c]	6.25	431	413 (10), 341 (30), 311 (100)	283 (100)	235 (10), 239 (100), 224 (20), 196 (30), 183 (50)
18	Apigenin 6-*C*-hexoside	6.49	431	413 (5), 383 (5), 341 (30), 311 (100)	283 (100)	235 (25), 239 (100), 224 (40), 197 (80), 183 (60)
19	4′-*O*-Methylapigenin 8-*C*-hexoside	7.83	445	427 (5), 355 (20), 325 (100)	297 (65), 282 (100)	282 (50), 253 (100), 209 (80), 183 (20), 161 (60)

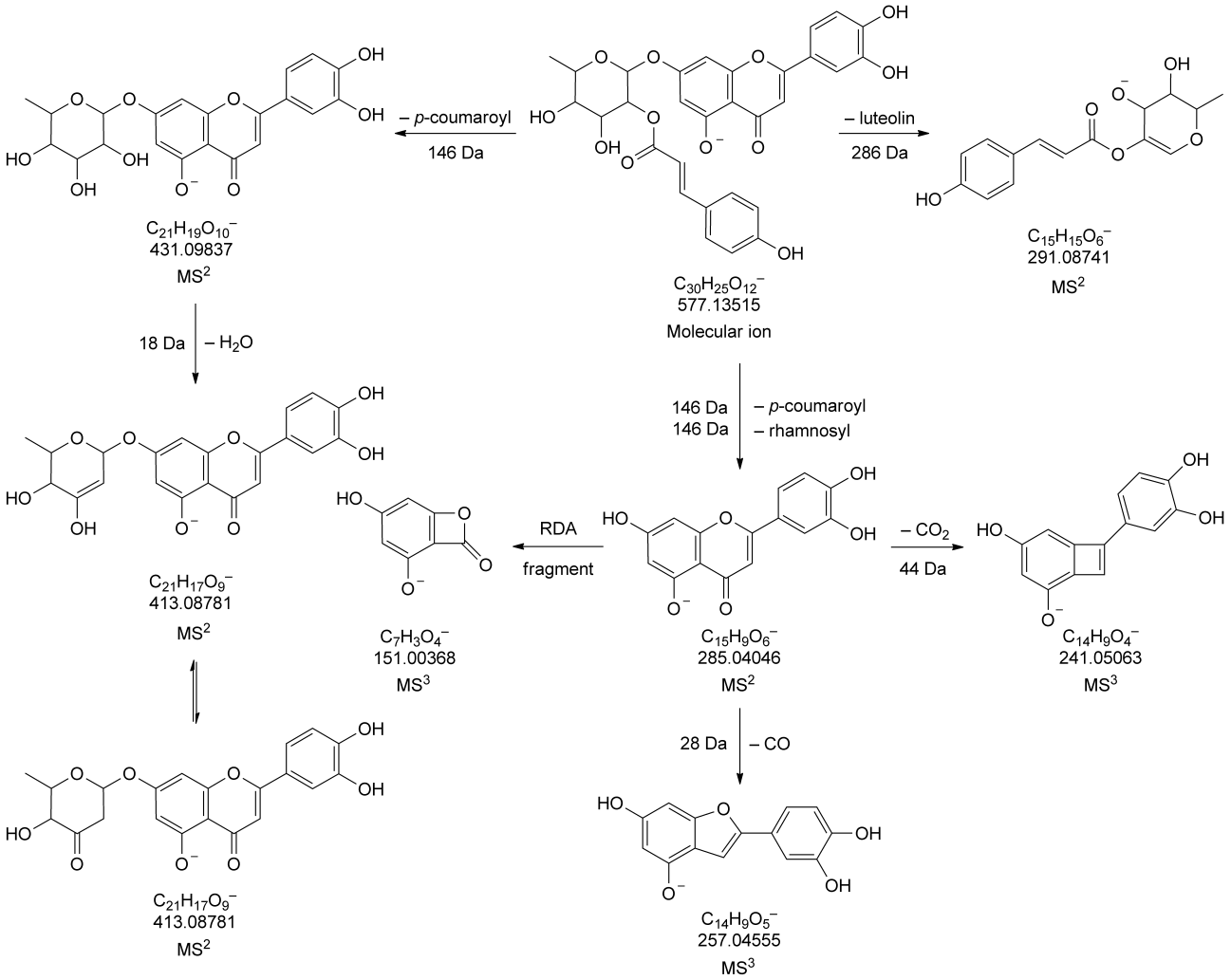
Table 2. (cont.)

Peak No.[a]	Compound name	t_R [min]	Parent ion [*m/z*]	MS^2 Fragments [*m/z*] (% base peak)	MS^3 Fragments [*m/z*] (% base peak)	MS^4 Fragments [*m/z*] (% base peak)
Xanthones						
20	4′-*O*-Methylapigenin 6-*C*-hexoside	8.00	445	409 (10), 355 (30), 325 (100)	297 (60), 282 (100)	282 (30), 253 (100), 211 (60), 189 (15), 162 (30)
Flavonoid *O*-glycosides						
21	Quercetin 3-*O*-galactoside[b][c]	6.43	463	302 (20), 301 (100), 300 (25)	272 (10), 257 (10), 193 (5), 179 (100), 151 (30)	–
22	Isorhamnetin 3-*O*-(2″-*O*-rhamnosyl)hexoside	6.51	623	592 (10), 503 (5), 459 (20), 315 (50), 314 (100)	299 (100), 285 (10), 271 (10)	271 (100), 255 (15), 243 (10), 227 (5)
23	Quercetin 3-*O*-glucoside[b][c]	6.61	463	302 (20), 301 (100), 300 (30)	272 (20), 256 (20), 229 (10), 179 (100), 151 (60)	–
24	Kaempferol 7-*O*-(6″-rhamnosyl)hexoside[b]	6.77	593	327 (5), 285 (100), 267 (5)	267 (70), 257 (100), 241 (30), 239 (20), 229 (70)	–
25	Irisdichotin B	6.85	493	465 (50), 351 (10), 331 (100), 303 (90), 246 (40)	303 (100)	288 (100), 270 (15), 254 (5), 205 (10), 165 (10)
26	Isorhamnetin 3-*O*-(6″-rhamnosyl)hexoside[b]	6.86	623	315 (100), 300 (20), 271 (10), 255 (5)	300 (100), 287 (5), 272 (5)	271 (100), 255 (50), 151 (5)
27	Kaempferol 3-*O*-galactoside	6.87	447	327 (20), 285 (99), 284 (100), 255 (20)	267 (40), 256 (100), 241 (30), 227 (40), 213 (80)	–
28	Isorhamnetin 3-*O*-galactoside[b]	6.95	477	357 (20), 315 (50), 314 (100), 300 (10), 285 (10)	300 (40), 285 (100), 271 (50), 257 (10), 243 (20)	270 (100)
29	Kaempferol 3-*O*-glucoside[c]	7.05	447	327 (10), 285 (60), 284 (100), 255 (15)	–	–
30	Isorhamnetin 3-*O*-glucoside[b][c]	7.16	477	357 (10), 315 (50), 314 (100), 300 (5), 285 (10)	300 (20), 285 (100), 271 (90), 257 (10), 243 (20)	270 (100)
31	Luteolin 7-*O*-(2″-*p*-coumaroyl)rhamnoside[b]	9.98	577	431 (10), 413 (5), 291 (5), 286 (10), 285 (100)	257 (90), 241 (100), 151 (15)	–
Isoflavonoids and derivatives						
32	Tectoridin	6.68	461	446 (5), 341 (5), 299 (100), 298 (10), 284 (10)	284 (100)	–
33	Iristectorin B	7.04	491	477 (20), 476 (100), 329 (10), 328 (20)	314 (15), 313 (100), 299 (5), 298 (20), 270 (10)	298 (100), 285 (50), 270 (30)
34	Iristectorigenin A 7-*O*-hexuronide[b]	7.20	505	485 (5), 459 (5), 329 (100), 314 (5), 274 (10)	315 (10), 314 (100)	300 (15), 299 (100), 285 (20)
35	Iristectorin A	7.25	491	477 (20), 476 (100), 329 (10), 328 (10), 314 (5)	314 (25), 313 (100), 299 (5), 298 (10), 269 (10)	298 (100), 285 (30), 270 (20)
36	Irilone 4′-*O*-(6″-hexosyl)hexoside	7.51	621	323 (50), 298 (25), 297 (100), 263 (20)	–	–

Table 2. (cont.)

Peak No.[a]	Compound name	t_R [min]	Parent ion [*m/z*]	MS^2 Fragments [*m/z*] (% base peak)	MS^3 Fragments [*m/z*] (% base peak)	MS^4 Fragments [*m/z*] (% base peak)
Xanthones						
37	3′-Hydroxyltectoridin	7.54	477	417 (100), 345 (10), 315 (50), 272 (5)	402 (100)	385 (60), 368 (15), 342 (100), 314 (70), 286 (40)
38	Iridin	7.55	521	506 (15), 360 (20), 359 (100), 344 (20), 329 (10)	344 (100), 329 (5)	329 (100)
39	Dichotomitin 3′-*O*-(6″-hexosyl)hexoside[b]	7.70	681	358 (70), 357 (100), 323 (70)	–	–
40	7-*O*-Methyltectorigenin 4′-*O*-(6″-hexosyl) hexoside	7.77	637	313 (100), 299 (20)	298 (100)	283 (100), 255 (10)
41	Nigracin 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside[b]	8.02	647	585 (10), 545 (10), 342 (20), 341 (100)	326 (100)	311 (100), 298 (5), 283 (10), 269 (15)
42	Irifloside	8.13	489	327 (100)	312 (100)	284 (100), 256 (20), 179 (10)
43	Irilone 4′-*O*-hexoside	8.18	459	297 (100)	269 (100), 241 (40), 255 (30), 204 (30), 147 (60)	–
44	Irisolidone 7-*O*-hexoside	8.56	475	355 (10), 313 (100), 298 (5)	298 (100)	–
45	Tectorigenin	8.60	299	284 (100)	256 (100), 240 (70), 227 (90), 211 (30), 158 (30)	–
46	Dichotomitin 3′-*O*-hexoside	8.64	519	475 (10), 358 (30), 357 (100), 312 (5), 259 (10)	342 (100), 328 (10), 314 (5)	–
47	Irilone 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside[b]	8.71	603	541 (10), 459 (15), 441 (10), 297 (100)	269 (100), 251 (15), 241 (10), 227 (30), 176 (50)	–
48	Iristectorigenin A	9.79	329	315 (20), 314 (100), 311 (5), 293 (10), 171 (20)	299 (100), 284 (5), 271 (15), 255 (10), 227 (5)	271 (100), 255 (20), 243 (5), 227 (10), 199 (5)
49	Irigenin	9.93	359	345 (15), 344 (100)	329 (100), 326 (10), 314 (5)	314 (100), 311 (5), 301 (50), 298 (10), 285 (10)
50	Irilone	11.08	297	269 (100), 251 (10), 241 (10), 228 (10), 211 (10)	–	–
51	Iriflogenin	11.34	327	312 (100), 284 (5)	284 (100), 256 (15), 227 (10), 200 (5), 179 (10)	256 (100), 227 (60), 212 (10), 200 (20), 158 (15)
52	Irisolidone	11.80	313	299 (15), 298 (100), 294 (10), 267 (10)	283 (100), 255 (15), 228 (5), 211 (5), 199 (5)	255 (100), 239 (7), 211 (40), 195 (25), 159 (5)

[a] Peak numbers corresponding to *Figure 1*; t_R - retention time. [b] Identified in some of *Iris* sp. for the first time. [c] Confirmed using available standards. All the other compounds were identified based on MS data. [d] – stands for not detected fragments.



Scheme 1. Proposed fragmentation pathway of compound **31** (luteolin 7-*O*-(2″-*p*-coumaroyl)rhamnoside).

which corresponds to the fragmentation of kaempferol. This compound, kaempferol 7-*O*-(6″-rhamnosyl) hexoside, was characteristic for flowers of all three investigated *Iris* species. Irisdichotin B (compound **25**), eluted at 6.85 min with molecular ion at 493 *m/z*, was confirmed by examination of its MS data. It is well-known that this compound is specific to *Iris* sp. because it was previously identified in the *I. dichotoma* rhizome.[40] Compound **31** at 9.98 min, with molecular ion at 577 *m/z* and MS^2 base peak at 285 *m/z* (mass of deprotonated luteolin, obtained by elimination of 292 Da corresponding to *p*-coumaroyl (146 Da) + rhamnosyl (146 Da) residue) was tentatively identified as luteolin 7-*O*-(2″-*p*-coumaroyl)rhamnoside. MS^3 spectrum with base peak at 241 *m/z* confirmed the presence of luteolin as aglycone. Proposed fragmentation pathway of compound **31** is depicted in *Scheme 1*.

Isoflavones and Their Derivatives

Isoflavones and their glycosides are the main classes of polyphenolic compounds found in *Iris* species.[3] Many isoflavones were named after the type of *Iris* from which they were common or firstly isolated. Identification of isoflavones and their derivatives, in the absence of standards, was achieved using the available literature on phytochemicals previously isolated or just identified in some of *Iris* spp,[5,7,13,30,41–43] as well as by studying of its MS fragmentation pattern (exact mass and MS^4 fragmentation). *Tables 1* and *2* summarized MS data for all isoflavone derivatives (compounds **32–52**) found in our *Iris* species. Bearing

in mind that most of these compounds are already known to be present in *Iris* species, this paragraph will only give a brief overview of the identification of compounds that have not been identified so far in the aforementioned plant species. Thus, compound **34** (7.20 min; 505 *m/z*) generated MS^2 base peak at 329 *m/z* resulting by the loss of hexuronic acid residue moiety (176 Da). MS^3 spectrum showed base peak at 314 *m/z* (generated by elimination of methyl group) and this compound was marked as iristectorigenin A 7-*O*-hexuronide. Iristectorigenin A (compound **48**), known to be present in *I. tectorum*,[42] was also identified in the test samples. Compound **39** (found only in *I. humilis* rhizome) at 7.70 min and molecular ion at 681 *m/z* was identified as dichotomitin 3′-*O*-(6″-hexosyl)hexoside. It produced MS^2 base peak at 357 *m/z*, corresponding to the mass of deprotonated dichotomitin. Dichotomitin 3′-*O*-hexoside (compound **46**) was also identified only in *I. humilis* rhizome, and its fragmentation was confirmed by available literature.[13] Nigracin, known to be present in extracts of *I. germanica* and *I. pallid*,[7] in this study it was not found in the form of aglycone, but only glycoside and it was marked as nigracin 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside (**41**). In the literature, there is no known case of the presence of isoflavone derivatives with 3-hydroxy-3-methylglutaryl group, but this structure is proposed as the most logical, because it fits into exact mass and MS fragmentation. Similar to that, peak **47** eluted at 8.71 min with 647 *m/z* (MS^2 base peak fragment at 297 *m/z* and MS^3 base peak fragment at 269 *m/z*) was tentatively identified as irilone 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside. It was only found in above-ground vegetative parts of *I. pumila*. Detailed fragmentation pathway proposed for compound 47 is shown in *Scheme 2*.

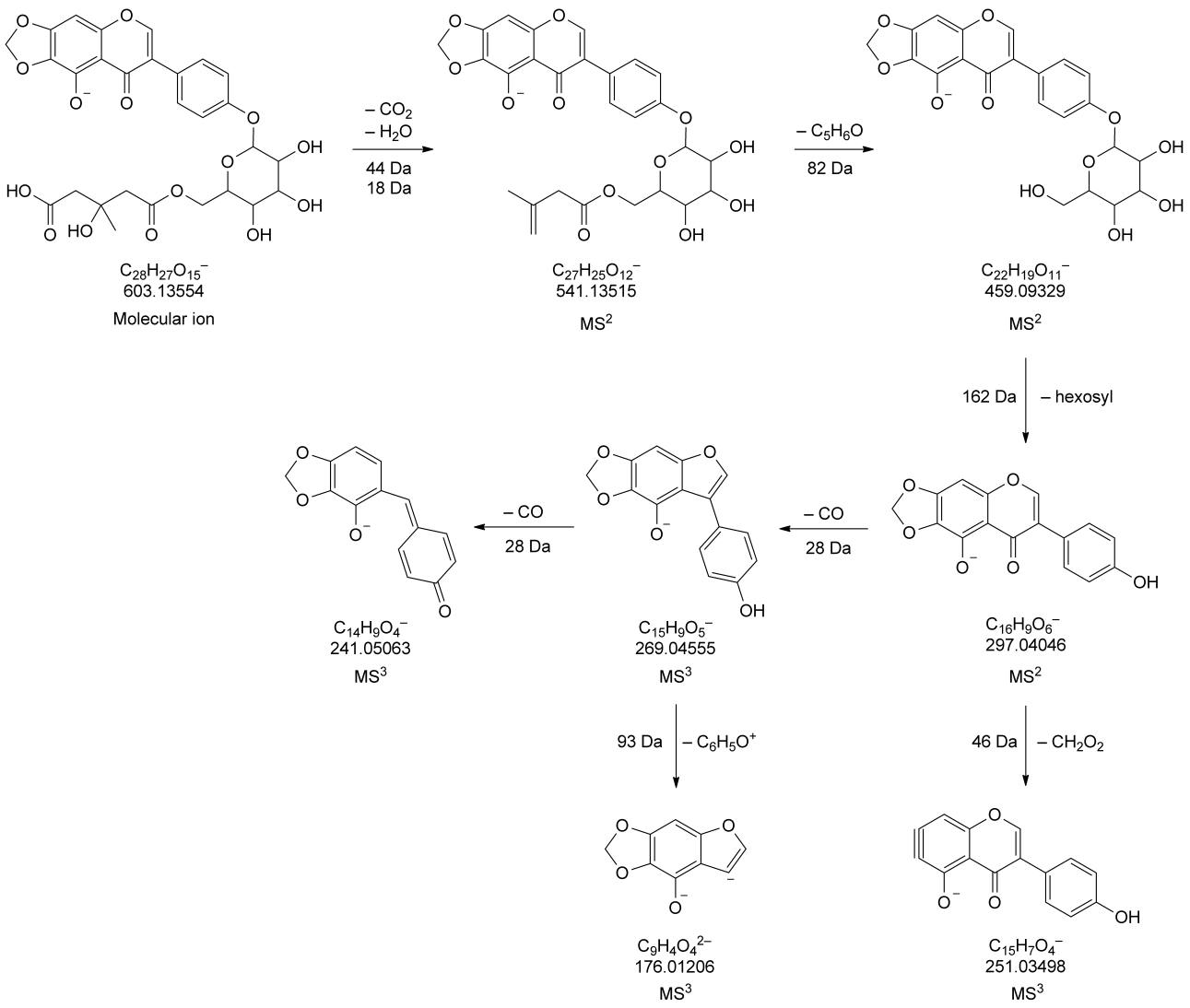
Chlorophylls and Carotenoids Content

Content of photosynthetic pigments (chlorophylls A and B) and total carotenoids in plant materials is shown in *Table 3*. Significant differences in the content of these pigments were recorded in the analyzed plant parts. Expectedly, the highest chlorophyll content has been detected in above-ground vegetative parts of *Iris* plants, a significantly lower content of these pigments was observed in flowers, while presence of both chlorophylls has not been recorded in underground part of plants – rhizomes. High positive correlation was found between the content of chlorophyll A and chlorophyll B ($r = 0.95$) and chlorophyll A/chlorophyll B and carotenoids ($r = 0.87$, $r = 0.80$, respectively). Results

Table 3. Content of phytochemicals, soluble sugars, starch and Trolox equivalent antioxidant capacity (TEAC) in different parts of *Iris* species expressed on dry weight (DW).

	R_1[a] [b]	R_2	R_3	AGP_1	AGP_2	AGP_3	F_1	F_2	F_3
Chlorophyll A (μg/g of DW)	n.d.[c]	n.d.	n.d.	217.3 ± 0.1	603.3 ± 0.1	388.2 ± 0.8	9.8 ± 0.1	32.3 ± 0.3	38.0 ± 0.3
Chlorophyll B (μg/g of DW)	n.d.	n.d.	n.d.	45.6 ± 1.0	136.7 ± 1.1	88.4 ± 2.6	4.6 ± 0.1	53.2 ± 1.8	n.d.
Carotenoids (μg/g of DW)	n.d.	0.92 ± 0.01	n.d.	70.3 ± 0.1	165.7 ± 0.3	102.9 ± 1.0	83.8 ± 0.5	21.5 ± 1.3	61.1 ± 1.2
Total phenolics (mg GAE/g of DW)	13.8 ± 0.6g	8.8 ± 0.6b,f	11.2 ± 0.4d	11.0 ± 0.7d,h	9.3 ± 0.4c,e	8.4 ± 0.6a,b,c	12.8 ± 0.8g	9.8 ± 0.6e,f,h	7.4 ± 0.5a
Total flavonoids (mg QE/g of DW)	2.9 ± 0.2a	0.98 ± 0.06b	1.04 ± 0.07b	4.0 ± 0.2	2.8 ± 0.2a	2.9 ± 0.2a	3.5 ± 0.2	1.74 ± 0.06	0.79 ± 0.03
Soluble sugars (mg/g of DW)	17.9 ± 0.8a	15.5 ± 0.5	9.9 ± 0.2	28.8 ± 0.8b	18.9 ± 0.8a	29.7 ± 0.7b	23.6 ± 0.7c	22.6 ± 0.9c	23.0 ± 1.1c
Starch (mg/g of DW)	37.4 ± 1.2	6.3 ± 0.5	12.0 ± 0.8	1.61 ± 0.06	2.3 ± 0.1	1.10 ± 0.06a	4.7 ± 0.3	1.17 ± 0.06a	1.10 ± 0.06a
TEAC (μmol Trolox/g)	178.3 ± 5.2a	148.2 ± 4.0b,c	80.4 ± 3.1	156.1 ± 4.8c	141.9 ± 3.0b	71.1 ± 2.8	184.4 ± 4.6a	110.7 ± 3.1	49.9 ± 2.5

[a] R- rhizome; AGP – above-ground vegetative parts; F – flower [b] 1 – *Iris variegata* L.; 2 – *Iris pumila* L.; 3 – *Iris humilis*. [c] n.d.– not detected. Data are presented as means ± SD. Means in the same row not sharing a letter are significantly different ($p < 0.05$).



Scheme 2. Proposed fragmentation pathway of compound **47** (irilone 4′-*O*-[6″-(3-hydroxy-3-methylglutaryl)]hexoside).

related to the content of chlorophylls are similar (AGP 2 and AGP 3) or lower (AGP 1) than results obtained for leaves and stems of different *Mentha* species.[44] In the case of carotenoids, the highest content was found in green parts of *I. pumila* (165.7 μg/g of dry weight) and *I. humilis* (102.9 μg/g of dry weight). Similar results were reported in the case of *Mentha* green parts.[44] Rhizomes of *Iris* species did not contain carotenoids except in the case of *I. pumila* rhizome (0.92 μg/g of dry weight).

Total Phenolic Content (TPC) *and Total Flavonoids Content* (TFC)

Total phenolic content (*Table 3*) in plant samples was ranged from 7.4 mg GAE/g of dry weight, which was found in flowers of *I. humilis*, to 13.8 mg GAE/g of dry weight presented in rhizomes of *I. variegata*. According to obtained results, analyzed samples can be compared to results obtained for 45 selected medicinal plants[45] with very similar the highest content found in plant *Smilax glabra* Roxb. (14.24 mg GAE/g). Furthermore, in two species (*Cynanchum atratum* Bunge and stem of *Lonicera japonica* Thunb.), TPC values (7.75 and 7.81 mg/g GAE DW) were in range of the lowest TPC for *Iris* species. Phenolic content in above-ground part of *I. pumila* was similar to result obtained for Tossa jute leaves.[46] In the case of flavonoids, a similar distribution was recorded as for total phenolic content – *I. variegata* possessed maximal amounts of flavonoids in green part (4.0 mg QE/g) while *I. humilis* flowers, again, have shown the

lowest flavonoids content (0.79 mg QE/g of dry weight). TFC in above-ground part of *I.* variegata was in accordance with results for ethanolic extract of *Corchorus olitorius* L. leave.[46] Determination of the amount of bioactive compounds, such as phenolics, flavonoids or terpenes, is important because of their further use. For instance, the presence of four different irones compounds (*cis*-α-irone, *trans*-α-irone, β-irone and *cis*-γ-irone) in *Iris* spp. represents the basis for application of their essential oils as perfumes components in cosmetic industries.[47] Furthermore, the application of plant tissue culture techniques, based on embryogenic callus and somatic embryos production, it is possible to produce the desirable quantity of plant metabolites and overcome the problems connected with *Iris* plants such as long cultivation period, difficulties to collect and rapid decline of population size.[47]

Soluble Sugars and Starch Content

According to obtained results (*Table 3*) for sugars content, *I. humilis* contains maximum (above-ground vegetative parts) and minimum (rhizomes) amounts of soluble sugars depending on plant part. In the case of starch, the lowest contents were found in leaves, stems and flowers of this species (1.10 – 1.17 mg/g). Rhizomes of *I. variegata* can be described as best 'reservoir' of starch with 37.4 mg of starch/g of dry weight. The remaining two rhizomes, also, showed increased content of starch, which is in accordance with the role of this part of the plant. Comparing to results of Ranwala and Miller,[48] soluble sugars content was slightly lower (rhizomes) or in range (above-ground vegetative parts and flowers) with results obtained for glucose, fructose and sucrose content in storage organs of four different *Iris* species (~30 mg/g DW) except in the case of species *I. xiphium* (~90 mg/g DW). On the other side, starch content was significantly lower than contents found in the same investigation (471 – 539 mg/g).

Total Antioxidant Capacity

One of the main advantages of applying the ABTS method compared to other antioxidant tests (such as DPPH) is that analysis can be performed at different pH levels and by using both aqueous or extracts prepared in some organic solvents.[49] This is important especially in the case of some phenolic compounds which are pH-sensitive such as anthocyanin pigments presented in *Iris* spp. flowers.[10] Further, in this investigation, the methanolic extracts were used because Shalaby and Shanab[49] have shown that methanol extracts of *Spirulina platensis* possessed the higher ABTS antioxidant activity compared to the aqueous ones. Total antioxidant capacity of plant extracts, expressed as Trolox equivalent antioxidant capacity (TEAC), was ranged from 49.9 to 184.4 μmol Trolox/g of dry weight (*Table 3*). These results are comparative with results for different medicinal plants.[45] The highest TEAC value was equal as results that obtained for species *Scutellaria baicalensis* Georgi.[45] Other thirteen plant species showed also TEAC values in similar range to analyzed three *Iris* species. Correlation analysis revealed that the significant positive correlation between TEAC and TPC ($r = 0.72$) existed whereas no correlation was found between TFC and TEAC. These results indicated that besides flavonoids other components present in extracts with reducing activity can contribute to the total antioxidant capacity of *Iris* extracts. These results were in accordance with findings of other authors[45] who demonstrated that the highest and the lowest TPC values are followed with highest and lowest TEAC values.

Conclusions

In this study, phytochemical analysis of three different *Iris* species was conducted. Detailed xanthones, flavonoid-*C*-glycosides, flavonoid-*O*-glycosides and isoflavones profiles of *I. humilis*, *I. pumila* and *I. variegata* were obtained by LC/MS analysis. In total, 52 different compounds were identified among which 9 compounds are reported for the first time. Plant rhizomes contained the largest number of identified compounds – both *I. pumila* and *I. variegata* rhizomes contain 25 different compounds. Analysis of *I. humilis* ssp. *arenaria* rhizome has shown presence of 18 phenolics. Above-ground vegetative parts and flowers of *Iris* sp. possessed between 6 and 18 compounds. All investigated samples have shown high content of phenolic compounds which is comparable with different medicinal plants. High antioxidative capacity, expressed through Trolox equivalent value, was determined. Given results for phenolic profile can be used as potential 'botanical fingerprint' for investigated *Iris* species while good results for TPC and TEAC classify selected *Iris* sp. as potentially applicable for medical or some industrial purposes. In addition, these findings could be useful for estimation of potential of *Iris*

Figure 2. Appearance of three *Iris* species: a) *I. pumila* L., b) *I. variegata* L., c) *I. humilis.*

species for production of plant metabolites by callus for pharmaceutical/cosmetics industries.

Experimental Section

General

Acetonitrile, formic acid (both MS grade), acetone, methanol (both HPLC grade), Folin-Ciocalteu reagent and phenolic standards were purchased from Sigma-Aldrich (Steinheim, Germany). Perchloric acid, aluminum chloride, sodium nitrite, sodium hydroxide and sodium carbonate were obtained from Zorka Pharma (Šabac, Serbia). Ultrapure water (ThermoFisher TKA MicroPure, 0.055 μS/cm) was used to prepare standard solutions and blanks. Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA). Three *Iris* species (*I. humilis*, *I. variegata* L., *Iris pumila* L.) investigated in the current study are presented in *Figure 2*.

In Serbia, populations of *I. humilis* (Sandy iris) were observed at only two sites in the protected areas: Subotica Sands and Selevenj heath, from where the plants were taken for the purpose of chemical analysis. This is a rhizomatous perennial species, with long thin rhizome, about 2–5 mm thick. Rhizome has many thickened branched nodes making clumps of plants. Leaves are grass-like (8–10 mm wide) and the stem is short. It blooms in April and May. There are one or two flowers per stem and they are pale yellow with thin purple veins and are fragrant (vanilla scented). Fruit are at the top of the stem. Flowering period is short and each flower lasts only one day. Plant specimens of *I. variegata* (Hungarian or variegated iris) and *I. pumila* (dwarf bearded iris) were taken from undisturbed natural populations growing in the Special Nature Reserve – Deliblato sands, the largest European continental sandy terrain located in the south-east part of the Pannonian Plain, in Serbia. *Iris variegata* L. is a perennial clonal herb while *Iris pumila* L. probably originated as a natural hybrid between *I. pseudopumila* Boissier & Heldreich and *I. attica* Tineo. Variegated iris grows up to 1 m high and has stout rhizome with roots that can go up to 10 cm deep in the ground. Leaves are dark green, ribbed, around 2–3 cm wide. Usually, there are 2–5 big flowers per stem. The scentless flowers appear in early summer, May–June. The flowers are yellowish-white with different networks of brown-purple veining on the falls. Contrary to other two *Iris* species in this research, *I. pumila* exhibits huge flower color genetic polymorphism (yellow, purple, violet, blue, cream and white). Fruits are at the bottom of the stem. From a very similar *I. humilis*, it distinguishes with this fruit feature and also slightly broader leaves (up to 20 mm). Dwarf bearded

iris is found growing along the forest edges at sun-exposed open sites, unlike *I. variegata* that inhabits almost equally often sun exposed and understory sites.[50] The collection of biomass samples was made from its natural habitats in Serbia:

I. humilis: Selevenj heath, protected area N 46°08′67″, E 19°55′17″ at 87 m a.s.l.;

I. pumila: Deliblato sands, protected area N 44°57′36″, E 21°02′08″ at 157 m a.s.l.;

I. variegata: Deliblato sands, protected area N 44°57′48″, E 21°02′54″ at 148 m a.s.l.;

Minimal amount of biomass were sampled for the purpose of chemical analysis because these *Iris* species are endangered and protected. Since they are rhizomatous perennial herb, the rest of each sampled plant were preserved in natural habitat and labeled for further analysis. Plant specimens were collected and identified by Dr. S. Avramov (Institute for Biological Research, Siniša Stanković, Serbia) during May – July 2016. After excavation, plants were divided in three parts: rhizomes (R), above-ground vegetative parts (stem and leaf; AGP) and flowers (F). All parts were thoroughly washed, dried and after that cut into pieces, packed in plastic bags, vacuumed and placed at dark and cold place (−80 °C) until further analysis.

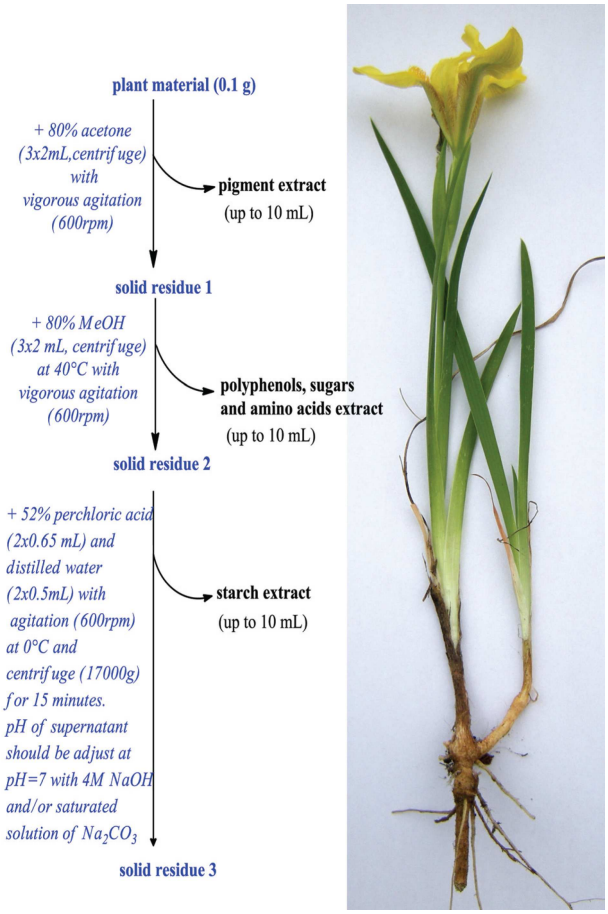


Figure 3. Extraction procedure used for separation of selected phytochemicals – pigments, total phenolics, total flavonoids, soluble sugars and starch.

Extraction of Plant Materials

Extraction procedure, based on Laware method,[51] is presented in *Figure 3*.

UHPLC-MS/MS Orbitrap Qualitative Analysis

Separations of compounds of interest were performed using an ultrahigh-performance liquid chromatography (UHPLC) system consisting of a quaternary Accela 600 pump and Accela autosampler (ThermoFisher Scientific, Bremen, Germany). The UHPLC system was coupled to a linear ion trap – orbitrap mass spectrometer (LTQ OrbiTrap MS) equipped with heated electrospray ionization probe (HESI-II, ThermoFisher Scientific, Bremen, Germany) in negative ion mode. A Syncronis C_{18} column (100×2.1 mm, 1.7 μm particle size) at 40 °C was used for compound separation. Flow rate was set at 0.250 mL/min and the mobile phase consisted of (A) water + 0.1 % formic acid and (B) acetonitrile. The injection volumes were 5 μl and linear gradient programs were as follows: 0.0 – 1.0 min 5 % (B), 1.0 – 14.0 min from 5 % to 95 % (B), 14.0 – 14.1 min from 95 % to 5 % (B) and 5 % (B) for 6 min.

Parameters of the ion source were as in literature.[52] The MS spectra were acquired by full-range acquisition covering 100 – 1000 *m/z*. Resolution was set to 30,000 for full scan analysis. The data-dependent MS/MS events were always performed on the most intense ions detected in the full scan MS. The ions of interest were isolated in the ion trap with an isolation width of 5 ppm and activated with 35 % collision energy levels. Settings of dynamic exclusion were as previously described.[53] Xcalibur software (version 2.1) was used for the instrument control, data acquisition and data analysis.

Determination of Pigment Content

During the first step of subsequent extraction procedure, the obtained acetone extract contains three different photosynthetic pigments: chlorophylls A and B and carotenoids of which the content was determined by spectrophotometric method.[51] Results for pigments content are expressed as μg/g of dry weight samples.

Determination of Total Phenolic and Total Flavonoids Content

The second stage of extraction procedure produced 80% MeOH extract which contains phenolics compounds and flavonoids as important sub-fraction of phenolics. Determination of total phenolic content was conducted by application of standard Folin-Ciocalteu method[54] while total flavonoids were determined with aluminum chloride method.[55] All results for phenolic content are expressed as mg of gallic acid equivalent (GAE) per gram of dry weight of samples. The obtained results for total flavonoid content are expressed as milligrams of quercetin equivalents per gram of dry weight of samples (mgQE/g).

Determination of Soluble Sugars and Starch

Soluble sugars content and starch content (part of 80% MeOH extract) were determined by standard anthrone spectrophotometric method[56] using sugars and starch extracts generated after second and third steps of subsequent extraction procedure, respectively.

Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

Antioxidant activity of *Iris* extracts were determined applying method of Li et al.[45] using 1 mL of MeOH plant extracts and 20 mL of ABTS solution. Obtained results are expressed as μmol Trolox/g of dry weight of used plant materials.

Statistical Analysis

For determination of statistical parameters (mean values ± standard deviation), Duncan's multiple range test was applied ($p < 0.01$). The correlation analysis between pigments content, total phenolic content (TPC), total flavonoids content (TFC) and antioxidant activity (TEAC values) were performed and expressed through Pearson's coefficient (r). Correlations at $p < 0.05$ were considered as significant.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of the Republic Serbia, Project Nos. TR31069, OI172017, 173025 and 46009.

Author Contribution Statement

S.N.A. contributed in the collection and identification of plant material. A.Ž.K., U.M.G., M.B.P., S.P.S., M.B.B., M.P.M.J. and Ž.L.T. participated in experimental procedures, determinations and interpretation of the data. A.Ž.K., U.M.G. and S.N.A. participated in the conception and design of the manuscript. M.B.P., M.P.M.J. and Ž.L.T. contributed in the drafting and polishing the manuscript.

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Received October 25, 2018
Accepted January 4, 2019

