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Chemical Fingerprinting, Total Phenolics and Antioxidant Activity of Some *Iris* Taxa

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Abstract: Some *Iris* taxa covered in this study were used or are still being used as remedies in European folk medicine. The most common indication for the decoction of *Iris* rhizomes is sore throat and productive cough, especially in case of the taxon *I. domestica* (L.) Goldblatt & Mabb., the well-known remedy in traditional Chinese medicine. The aim of this study is to chemically characterize rhizomes of seven wild and medicinal *Iris* taxa and to assess the antioxidant activity of the rhizome extracts. The HPTLC chromatograms and densitometric spectrum scan of the spots were used for untargeted metabolic profiling and tentative metabolite class identification of *Iris* taxa. Total number of found spots is 41, three major spots are found in all taxa, and 22 spots are found to be taxon specific. Two xanthones, three isoflavonoid glycosides, 18 isoflavonoid aglycons (isoflavonoids), and five acetophenones/benzophenones are tentatively identified. The taxon *I. pallida* Lam. contains largest amount of phenolic substances (28.5 ± 2.0 mg GA_E / g dry plant material) and was the most effective ABTS scavenger (27.5 ± 2.4 mg $TROLOX_E$ / g dry plant material). The taxon *I. illyrica* Tomm. ex Vis. was the most effective in both DPPH and FRAP assays, 10.1 ± 1.5 and 17.3 ± 0.1 mg $TROLOX_E$ / g dry plant material, respectively. The most effective taxa in the metal chelating assay were *I. croatica* Horvat & M.D.Horvat (6.1 ± 0.1 mg $EDTA_E$ / g dry plant material) and *I. germanica* var. *florentina* (L.) Dykes (5.8 ± 0.1 mg $EDTA_E$ / g dry plant material). European *Iris* taxa have a relatively similar and partly shared chemical profile with Asian taxon *I. domestica*, which might be a sign of equivalence in the medicinal value.

Keywords: genus *Iris*, isoflavonoids, xanthones, free radicals, FRAP, metal chelation.

INTRODUCTION

RHIZOMES of taxa of the genus *Iris* L. have been used in Croatian traditional medicine, as well as throughout Northern hemisphere where other taxa of the very numerous genus *Iris* are well known medicinal herbs in their respective habitat.[1] Dioscorides described Illyrian iris in the compendium of medicinal plants - *De materia medica* from the 1st century AD, it was a tall bearded iris native to Dalmatia.[2] Modern monographs of *Iris* taxa state that the rhizomes of few tall bearded irises are used in traditional medicine but their use in modern phytotherapy is discouraged, since there were relatively few proofs of pharmacological activity at the time.[3] More recently, The Committee on Herbal Medicinal Products (HMPC) of European Medicines Agency (EMA) included Rhizoma Iridis and *I. germanica* rhizoma drugs to the Inventory of herbal substances for assessment (EMA/HMPC/494079/2007).

Historically, taxonomy of the genus was frequently misunderstood due to slight morphological differences between closely related taxa. German commission E monograph on orris root (Rhizoma Iridis) features taxon *I. pallida* Lam. as the dalmatian sweet iris[4], although the taxa from the series Pallidae native to Dalmatia are *I. pseudopallida* Trinajstić and *I. illyrica* Tomm. ex Vis.[5] Taxon *I. pallida* is considered to be a cultivated variety, traditionally cultivated in the Mediterranean area and elsewhere.[5] Three mentioned taxa, as well as taxon *I. germanica* var. *florentina* (L.) Dykes, which is also considered as the valid plant material for Rhizoma Iridis[4], are included in this study. Another endemic tall bearded iris, *I. croatica* Horvat & M. D. Horvat, and short-stemmed bearded *I. adriatica* Trinajstić ex Mitić, are also tested due to the phylogenetic proximity to the medicinal taxa, which can affect their chemical composition.

Chemical fingerprint obtained via high performance thin layer chromatography (HPTLC) can be useful for differentiating similar species by their extract composition and quality control of the plant material.[6] Applied separation techniques offer an opportunity for qualitative and semi-quantitative analysis of pharmaceutically significant compounds and chemotaxonomic marker compounds for rapid species determination can be found.[7] Phenolics tend to have chemotaxonomic significance in the genus *Iris*, particularly xanthones, isoflavonoids, acetophenones, and benzophenones.[8] Furthermore, some of those compounds possess promising pharmacological activity such as anti-inflammatory, antidiabetic, bacterial resistance modulating, chemoprotective and phytoestrogenic.[9,10]

Rhizome of Blackberry Lily, Belamcandae chinensis rhizoma (Ph. Eur.), is the plant material derived from the taxon *I. domestica* Goldblatt & Mabb. (syn. *Belamcanda chinensis* (L.) DC.). It is used in traditional Chinese medicine (TCM) as an antipyretic, expectorant, mucolytic, carminative, purgative, and for other uses.[11] Extracts of *I. domestica* rhizomes, as well as some isolated compounds, exhibit significant antioxidant activity and the taxon is considered to be a moderate scavenger of free radicals and active oxygen species in TCM.[12] Furthermore, the Croatian endemic taxa have not been extensively studied for phytochemical compounds and activities. Thus, the objective of this study is to analyze chemical fingerprints of endemic wild and cultivated *Iris* taxa, along with commercial plant material, by HPTLC, as well as to measure total phenolics content and antioxidant activity (antiradical activity, ferric reducing power and metal chelating) of the plant extracts.

EXPERIMENTAL SECTION

Plant Material and Chemicals

The rhizomes of wild taxa were collected as follows: *I. adriatica* on the island Brač (43°21'30.3" N, 16°35'49.6" E) and voucher specimens are kept in the herbarium of the Department of Biology, Faculty of Science, University of Zagreb, Croatia; *I. illyrica* on the island Vir (44°18'27.3" N, 15°1'52.5" E); *I. pseudopallida* on the mountain Biokovo (43°19'58.4" N, 16°59'2.4" E) and *I. croatica* on the mountain Papuk (45°29'35.5" N, 17°51'3.2" E). Specimen of taxon *I. pallida* was collected in Pharmaceutical Botanical Garden *Fran Kušan* in Zagreb. Voucher specimens are kept in the herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Medicinal taxa were acquired commercially, *I. germanica* var. *florentina* from the supplier Youherbit (Patras, Greece), and *I. domestica* on the street market in Bangkok (Thailand).

Methanol (HPLC gradient grade) for the extraction was purchased from VWR (Radnor, Pennsylvania, SAD). Solvents for the thin layer chromatography were acquired from following suppliers: methanol, chloroform and toluene from Carlo Erba Reagents (Barcelona, Spain) and formic acid from Scharlau (Barcelona, Spain). Sodium carbonate, sodium acetate, ethylenediaminetetraacetic acid (EDTA) disodium salt and the Folin–Ciocâlteu reagent were purchased from Kemika (Zagreb, Croatia). Positive controls for spectrophotometric measurement, gallic acid (GA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) were purchased from Acros Organics (Pittsburgh, USA). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate (ferrozine), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron(III) chloride anhydrous and iron(II) chloride tetrahydrate were acquired from Merck KGaA (Darmstadt, Germany). Hydrochloric acid was purchased from Lach-Ner (Neratovice, Czech Republic) and acetic acid from Avantor (Radnor, Pennsylvania, SAD).

Extract Preparation

The rhizomes were freeze dried, grinded and passed through sieve (0.315 mm). Powdered rhizomes were mixed with diatomaceous earth and covered with sea sand; mixture was extracted in the Dionex accelerated solvent extractor (Thermo Fisher Scientific, Waltham, Massachusetts USA), using methanol in three cycles of 5 min heating, followed by static period and purging under pressure of 69 bar and heating temperature of 68 °C. The extracts were centrifuged and set to exact volume with methanol for later quantification purposes.

HPTLC chemical fingerprinting

30 µL of the methanol extract was spray applied in nitrogen current on silicagel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany) for thin layer chromatography using semi-automatic apparatus, Linomat 5 (Camag, Muttenz, Switzerland). Mobile phase consisted of the mixture of solvents: toluene, chloroform, methanol and formic acid in the following ratio: 1:7:1:1 (*V*/*V*/*V*/*V*).[11] After the plate had been developed, the densitometric analysis was carried out using TLC Scanner 3 (Camag, Muttenz, Switzerland). Firstly, the absorption of emitted light (λ = 260 nm) was detected throughout the sample tracks. The integration of the peaks was performed automatically, using following settings: minimum slope = 10; minimum height = 10 AU; minimal area = 40 AU. Secondly, the absorption spectra of UV-Vis light were recorded for all found spots. Untargeted metabolic profiling was conducted by associating detected peaks into assigned spots based on similarity in retention time, with ± 0.03 tolerance in the

retention factor (R_F), while wavelength of the absorption maximum (λ_{max}) and the shape of absorption spectra were taken into count as the inclusion criteria for the clustering of the peaks into the spots assigned with numbers in increasing fashion, according to their respective R_F. UV-Vis absorption spectra were used for tentative determination of the class of the natural products, and in some cases the identity is proposed based on literature data.[11,12] Finally, the table noting the presence of particular peaks in the studied taxa is provided. Developed plates were photographed with camera (Canon, Tokyo, Japan) in the plate illuminator, Reprostar 3 (Camag, Muttenz, Switzerland).

Total Phenolics

Content of phenolic compounds was determined with the method according to Singleton and Rossi[14] using the UV-Vis Spectrophotometer (LG instruments, Leicestershire, UK). 1.25 mL of Folin- Ciocâlteu reagent was mixed with 125 µL of the extracts; 1 mL of 10 % Na_2CO_3 solution was added after 5 minutes. Absorbance of the solution was measured on 765 nm after an hour of incubation. All experiments were run in triplicate. Gallic acid was used to obtain calibration curve; results are expressed as milligram equivalents of gallic acid per gram of dried plant material (mg GA_E / g DM).

Antiradical Activity

Two stabile radicals were used to determine antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical. Solutions of the both free radicals were prepared and diluted until the absorbance equaled to 0.70 ± 0.05. The ABTS radical absorbance was monitored using wavelength of 734 nm,[15] while 517 nm was used for DPPH radical monitoring.[16] Decrease in absorbance was noted after addition of 10 µL of the extracts to 2 mL of the radical solution; incubation was carried on at room temperature, 1 minute for the ABTS assay and 30 minutes for the DPPH assay. Calibration curve was constructed using TROLOX, all measuring was done in triplicate and the results are expressed as milligram equivalents of TROLOX per gram of dried plant material.

Ferric Reducing Power Assay

The reagent for Ferric reducing power assay was made by mixing the following solutions: 20 mM $FeCl_3$, 10 mM TPTZ in 40 mM HCl, and 0.3 M acetic buffer (pH = 3.6), in the given ratio, 1:1:10 *(V/V/V)*.[17] Reaction is initiated in 2 mL of reagent with addition of 100 µL of plant extracts or TROLOX standard solution, which was used for calibration curve formation. The mixture was incubated for a period of half an hour in water bath at 37 °C, afterwards, the absorbance (λ = 583 nm) was measured. All measurements were done in triplicate and the results are expressed as milligram equivalents of TROLOX per gram of dried plant material (mg $TROLOX_E$ / g DM).

Metal Chelating Assay

The diluted extracts, 2.0 mL, were mixed with 0.05 mL 2 mM $FeCl_2$ solution and the reaction is initiated with addition of 0.2 mL of ferrozine (5 mM).[17] Absorbance (λ = 562 nm) of the samples was measured after 10 minutes of incubation on room conditions. EDTA was used as the positive control for calibration curve, therefore the results are expressed as milligram equivalents of EDTA per gram of dried plant material (mg $EDTA_E$ / g DM). All measurements were done in triplicate.

Statistical Analysis

Two statistical methods were applied on the chromatographic data using XLSTAT software (Addinsoft, Paris, France): principal component analysis (PCA) and agglomerative hierarchical clustering (AHC). The variables consisted of data pairs: the retention factor (R_F) and A_{260} calculated as densitometric peak area. Data was normalized with the assumption that no peak area is distributed throughout more than one spot. Therefore, nulling of densitometric peak area contribution might occur if measured absorbance contributes to another spot's peak area. The results of spectrophotometric measurement are expressed as mean ± standard deviation. Pearson correlation coefficient between the measured parameters was calculated and heatmap representing the correlation matrix is formed using JASP software (JASP, Amsterdam, Netherlands).

RESULTS AND DISCUSSION

HPTLC Chemical Fingerprinting

TLC is readily available technique for screening of plant materials. Based on retention factor and color (with and without derivatization) of the spot, substance can easily be identified when compared to standard. Enhanced with densitometry that provides UV-Vis analysis of the spot, TLC can be used for substance classification. Previous TLC studies show that mobile phase consisted of the mixture of solvents: toluene, chloroform, methanol and formic acid (1:7:1:1, *V/V/V/V*) is most appropriate for screening *Iris* taxa for normal phase chromatography on silica gel plates.[11]

The developed chromatographic plates were photographed while exposed to UV light (λ = 254 nm and λ = 366 nm). Lines quenching fluorescence of the green indicator bound to silica gel can be seen on plate exposed

to UV light λ = 254 nm, while on λ = 366 nm exposure, different fluorescence zones can be observed, most notably light blue, blue-green, grey and orange fluorescence (Figure 1). The chromatograms obtained by densitometric scan can be seen in Figure 1; the spot numbers are assigned to their respective peaks. Total number of peaks clustered into spots is 41, out of which 22 spots are found to be taxon specific, while 3 spots with significant peak area are found in all examined taxa in this study. Due to the limitations of the data acquiring technique, the precise identification of compounds was not the aim of this study. However, the class of the natural product could be assigned to 29 spots, while 12 spots were left unidentified (Table 1). Xanthones were tentatively identified based on characteristic absorption bands at 230–245 nm, 250–265 nm, 305–330 nm and 340–400 nm;[8] isoflavonoids are characterized by absorption maxima at 260–272 nm and shoulder peak at 310–340 nm;[12,18] and acetophenones/benzophenones by characteristic shape of the UV spectra and their absorption maxima on the shorter wavelengths comparing to the other two classes of compounds.[11] Results of untargeted metabolic profiling, chromatographic spot description and occurrence in particular taxa is provided in the Table 1.

The chromatographic spots 15, 25, 31 and 36 are found to be specific for *I. pallida*; 9, 18 and 30 are specific for *I. pseudopallida*; 2, 34 and 38 for *I. illyrica*; 12, 19 and 29 for *I. adriatica*; 24 and 35 for *I. germanica* var. *florentina*; 8, 13, 16, 23 and 33 for *I. croatica*; 7, 22 and 39 for *I. domestica*. The spots 5, 26 and 28 are found in all samples and are the most abundant based on their combined peak area percentage: 35.62 % for *I. pallida*; 28.56 % for *I. pseudopallida*; 27.93 % for *I. illyrica*; 39.38 % for *I. adriatica*; 32.42 % for *I. germanica* var. *florentina*; 24.47 % for *I. croatica*; and 39.93 % for *I. domestica*.

Tentative identification of the chemical nature of the spots led to identifying two spots exhibiting orange fluorescence formed by xanthones; three spots formed mainly by isoflavonoid glycosides; 18 spots formed by isoflavonoid aglycons (isoflavonoids), five spots formed by acetophenones or benzophenones, one spot formed by resveratrol, and 12 spots that are left unidentified due to uncertainties in classification. Spot 5 most probably consists of iridin and/or tectoridin, the two most common isoflavonoid glycosides in the genus, whose ratio can be variable even within a single taxon.[11,13] Spot 28 probably corresponds to irigenin, the most abundant isoflavonoid aglycon in the genus *Iris*.[13] Spot 26, likely resveratrol, together with spots 5 and 28, contributes from 24.47 up to 39.93 % of the total peak area. According to Wagner *et al.*[11] taxon *I. tectorum* Maxim., known adulterant and in some cases the substitute for *I. domestica* rhizomes, can be distinguished from *I. domestica* only by high acetophenone content and the absence of resveratrol. Thus, making the spot 26, assumedly resveratrol,[11] impractical for differentiation of European iris taxa from TCM taxon *I. domestica*. On the other hand, resveratrol was not detected in experiment using liquid chromatography coupled with mass spectrometry in the extracts of the taxon *I. adriatica* and the spot 26 might not correspond to resveratrol in all the examined taxa.[8] Other two major peaks – the spots 5 and 28, assumedly iridin, tectoridin and irigenin, also do not seem to be practical for taxa differentiation. European Pharmacopoeia (Ph. Eur.) suggests using an isoflavonoid, irisflorentin, as the reference

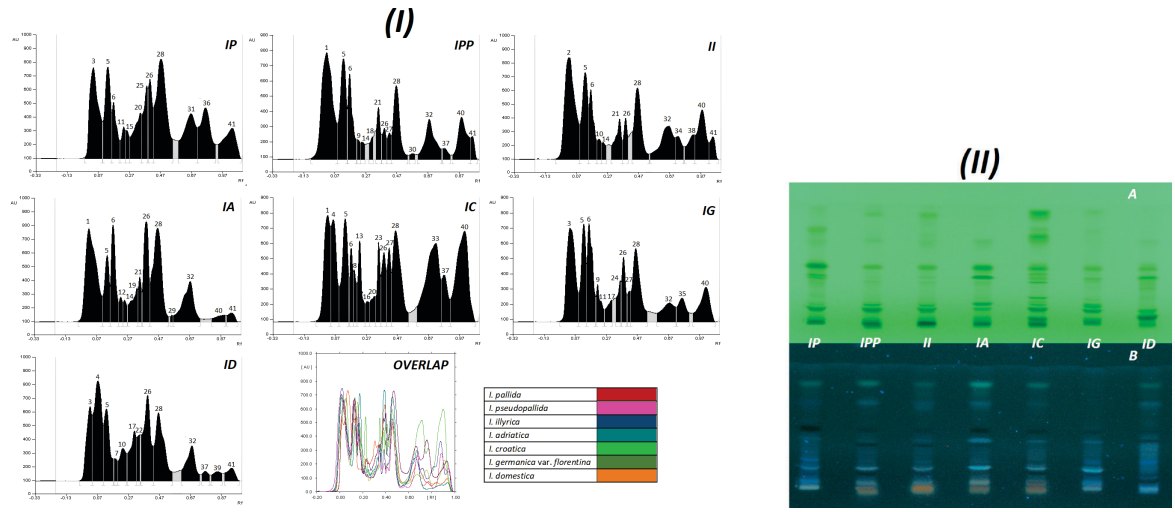

Figure 1. *(I)* HPTLC chromatograms with assigned peaks acquired by 260 nm wavelength absorption densitometric scan of the sample tracks (methanolic extracts); an overlap presented in colored chromatograms and color legend; *(II)* developed TLC plates under exposure to UV light; A) λ = 254 nm B) λ = 356 nm; *IP* – *I. pallida*; *IPP* – *I. pseudopallida*; *II* – *I. illyrica*; *IA* – *I. adriatica*; *IC* – *I. croatica*; *IG* – *I. germanica* var. *florentina*; *ID* – *I. domestica*.

Table 1. Untargeted metabolic profiling and tentative metabolite class identification of *Iris* taxa extracts using HPTLC separation and densitometric spectrum scan of the spots

#	R_F	λ_{max}	Class of the compounds	Occurrence in the taxa						
				IP	IPP	II	IA	IC	IF	ID
1	0.02	267	Xanthone(s)		*		*	*		
2	0.02	263	Xanthone(s)			*				
3	0.03	272	Isoflavonoid glycoside(s)	*					*	*
4	0.05	269	Isoflavonoid glycoside(s)					*		*
5	0.12	270	Isoflavonoid glycoside(s); idirin and tectoridin[10]	*	*	*	*	*	*	*
6	0.17	267	Isoflavonoid(s)	*	*	*	*	*	*	
7	0.18	288	N.I.							*
8	0.19	271	Isoflavonoid(s)					*		
9	0.21	313	N.I.		*					
10	0.22	321	N.I.			*				*
11	0.22	326	N.I.	*					*	
12	0.22	275	Isoflavonoid(s)				*			
13	0.22	268	Isoflavonoid(s)					*		
14	0.24	314	N.I.		*	*	*			
15	0.25	268	Isoflavonoid(s)	*						
16	0.27	321	N.I.					*		
17	0.30	265, 267	Isoflavonoid(s)						*	*
18	0.31	306	N.I.		*					
19	0.33	288	N.I.				*			
20	0.34	269	Isoflavonoid(s)	*				*		
21	0.34	314	N.I.		*	*	*			
22	0.34	264	Isoflavonoid(s)							*
23	0.35	247	Acetophenone or benzophenone					*		
24	0.36	261	Isoflavonoid(s)						*	
25	0.38	248	Acetophenone or benzophenone	*						
26	0.39	265-270	Resveratrol[10]	*	*	*	*	*	*	*
27	0.41	264, 267	Isoflavonoid(s)		*			*	*	
28	0.46	268	Isoflavonoid(s); possibly irigenin[13]	*	*	*	*	*	*	*
29	0.55	288	N.I.				*			
30	0.57	264	Isoflavonoid(s)		*					
31	0.66	279	N.I.	*						
32	0.67	269, 270, 272	Isoflavonoid(s); irisflorentin[10]		*	*	*		*	*
33	0.71	247	Acetophenone or benzophenone					*		
34	0.72	264	Isoflavonoid(s)			*				
35	0.75	251	Acetophenone or benzophenone						*	
36	0.76	246	Acetophenone or benzophenone	*						
37	0.77	262	Isoflavonoid(s)		*			*		*
38	0.82	264	Isoflavonoid(s)			*				
39	0.84	268	Isoflavonoid(s)							*
40	0.90	261, 262, 263	Isoflavonoid(s)		*	*	*	*	*	
41	0.94	200	Isoflavonoid(s) and other hydrophobic compounds (N.I.)	*	*	*	*			*

IP – *I. pallida*; IPP – *I. pseudopallida*; II – *I. illyrica*; IA – *I. adriatica*; IC – *I. croatica*; IG – *I. germanica* var. *florentina*; ID – *I. domestica*.; N.I. – not identified

substance in the TLC examination of *I. domestica* rhizomes; spot 32, assumedly irisflorentin,[11] is detected in all taxa except *I. pallida* and *I. croatica*. Further analysis using the Ph. Eur. test for foreign species should be carried out to exclude European iris taxa as the potential adulterants of *I. domestica* rhizomes.

The PCA biplot is shown in Figure 2. Variables were formed in a manner that R_F was associated with respective

densitometric peak area for every spot (data from Table 1). Thereby, taking into a count the presence of the spot in the taxon, as well as the relative amount of compound present in the extract. Variables are depicted as vectors gravitating to the observations, *i.e.* the extracts derived data, in two dimensional PCA space and represents 46.91 % of the total variability in the data. Dendrogram is formed by agglomerative hierarchical clustering of the observations based on (dis)similarity in the data, Euclidean distance between all the data pairs. Both graphs are presented in Figure 2.

PCA vectors indicate importance of the particular spot in the differentiation of the samples. The spots present only in singular taxon are viewed as the most important variables for the differentiation, as was expected for 22 taxon specific spots. The spots 20, 27, 32 and 40 (spots correspond to isoflavonoids based on UV spectra), as well as three unidentified spots: 14, 21 and 41 are seemingly the most important shared peaks for the taxa differentiation. Taxa *I. pseudopallida* and *I. illyrica* were found to be similar to a certain degree in the AHC analysis, as they formed the cluster with the most similarity in the entire data. Similarity in the chemical composition was previously reported for leaf flavonoid pattern between the taxa.[19] According to some authors, taxonomy of the series *Pallidae* is still unresolved and some consider species *I. pseudopallida* and *I. illyrica* to be subspecies of the taxon *I. pallida*.[5] Analysis performed in this study found that *I. pallida* is clustered primarily with *I. adriatica*, showing greater similarity in the chemical composition than is the case with supposed subspecies *I. pseudopallida* and *I. illyrica*. Secondly, *I. pallida* was clustered with the other medicinal taxa: *I. germanica* var. *florentina* and *I. domestica*, which seems to be in accordance to their use in traditional medicine.[20]

Spectrophotometric Measurements of Polyphenolics and Antioxidant Capacity

Spectrophotometry assays provide rapid *in vitro* assessment of antioxidant capacity of herbal extracts. The results of spectrophotometric measurements of total phenolics, antiradical activity, ferric reducing power and metal chelating effect, expressed as milligram of appropriate equivalent per gram of dried plant material (DM), are given in the Table 2.

Taxon *I. pallida* was found to contain the largest amount of phenolic substances based on Folin- Ciocâlteu assay and was the most effective ATBS radical scavenger. Isoflavonoids and their glycosides, most notably iristectorigenin A and its isomers were found to contribute the most to the radical scavenging in the study with *Iris* taxa from TCM.[18] It might be the case that the largest amount of iristectorigenin isomers is found in the extracts of the taxon *I. pallida*. The large variability of hydroxylation and methoxylation patterns of Iridaceae type isoflavonoids has been reported, and the degree of methoxylation is negatively correlated with the antioxidant activity.[21] The hydroxyl group linked to 4' carbon atom of the isoflavonoid skeleton seems to be the most significant contributing factor for the antioxidant activity.[22] The most effective in FRAP assay and superior DPPH radical scavenger was found to be the extract of the taxon *I. illyrica*. Large peak area and intense fluorescence of the spot 2 might by significant for antioxidant activity, since it is formed of xanthones, well-known and potent antioxidants.[17] Strong metal chelating activity was found for the taxa *I. croatica* and *I. germanica* var. *florentina,* indicating presence of compounds that are able to form σ-bonds with metals. Potent metal chelating effect was previously described for the taxon *I. germanica* L., a close relative to the two most effective taxa in this study.[23]

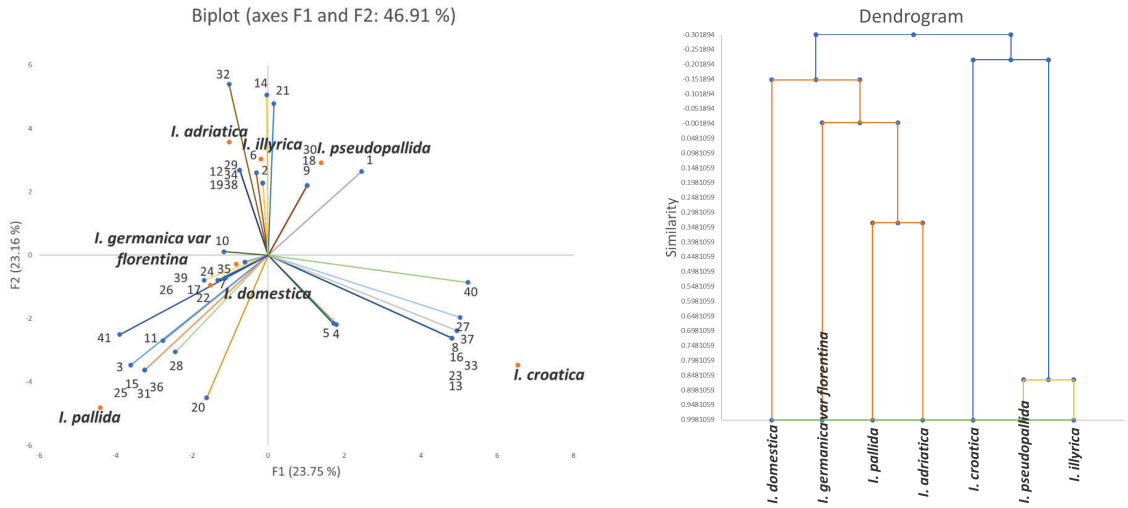

Figure 2. Principal component analysis biplot and agglomerative hierarchical clustering dendrogram derived from the statistical analysis of the chromatograms of *Iris* taxa extracts.

Table 2. Results of the spectrophotometric measuring of the total phenolics and the antioxidant activity in the extracts of *Iris* taxa

	Total phenolics	Antiradical effect – ABTS	Antiradical effect – DPPH	Ferric reducing power	Metal chelation
	mg GA_E / g DM	mg $TROLOX_E$ / g DM			mg $EDTA_E$ / g DM
I. pallida	28.5 ± 2.0	27.5 ± 2.4	2.6 ± 0.8	15.9 ± 0.9	3.8 ± 0.1
I. pseudopallida	19.8 ± 1.4	26.2 ± 1.4	3.6 ± 1.4	10.9 ± 0.6	5.3 ± 0.2
I. illyrica	22.2 ± 1.3	27.2 ± 2.6	10.1 ± 1.5	17.3 ± 0.1	4.4 ± 0.1
I. adriatica	20.4 ± 0.6	25.6 ± 3.2	4.9 ± 0.5	15.4 ± 0.4	2.3 ± 0.1
I. croatica	23.5 ± 1.5	26.7 ± 1.3	4.2 ± 1.4	14.7 ± 0.6	6.1 ± 0.1
I. germanica var. *florentina*	12.7 ± 0.8	17.2 ± 0.8	2.2 ± 0.8	8.5 ± 0.6	5.8 ± 0.1
I. domestica	20.6 ± 2.0	22.2 ± 2.7	3.6 ± 1.0	13.1 ± 0.5	4.6 ± 0.1

DM – dry mass

The correlation between the measured parameters is visualized in the heatmap of Pearson's correlation coefficient (Figure 3).

Statistically significant correlation was found both between the antioxidant assays: FRAP and ABTS ($p < 0.01$); FRAP and DPPH ($p < 0.01$); and between the total phenolics and the antioxidant assays: ABTS ($p < 0.01$) and FRAP ($p < 0.05$). The correlation between two antiradical assays was found to be insignificant. Observed differences might be due to the steric hindrance of the DPPH radical,[24] that may account for lower TROLOX equivalent values and lack of correlation in the measured values for the extracts in the DPPH assay compared with the ABTS assay. Negative correlation, although statistically insignificant, was noted for FRAP and metal chelating assay. The phenomenon derives from the fact that ferrous binding compounds might outcompete TPTZ in FRAP assay and cause a decrease in the measured value.[23] No significant correlation was found between the metal chelation assay and the rest of the spectrophotometric measurements. Compounds that are able to bind ferrous ions might not exhibit the antiradical or reducing effect directly, but they might inhibit Fenton's reaction in the biological environment and potentially prevent the formation of the endogenic free radicals in the human body.[17]

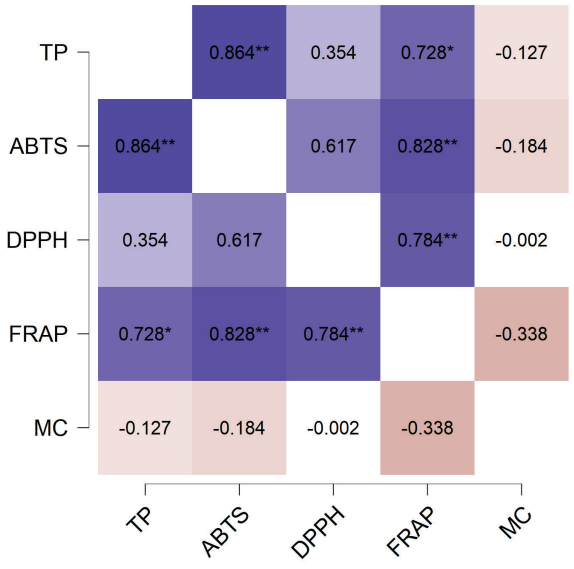

Figure 3. Pearson's correlation coefficient heatmap of the correlation between the measured parameters of *Iris* taxa extracts; * $p < 0.05$, ** $p < 0.01$; TP – total phenolics, ABTS and DPPH – antiradical assays, FRAP – ferric reducing power assay, MC – metal chelation.

CONCLUSION

HPTLC chemical fingerprints were obtained for seven wild and medicinal *Iris* taxa. 41 chromatographic peaks in the measured absorbance (λ = 260 nm) were observed, out of which, 22 peaks are taxon specific, while 3 peaks with significant peak area are present in all studied taxa. Two xanthones, three isoflavonoid glycosides, 18 isoflavonoid aglycons (isoflavonoids), five acetophenones/benzophenones and resveratrol were tentatively identified or classified. Compounds iridin, tectoridin, irigenin and resveratrol were identified based on previously reported HPTLC fingerprinting method for *Iris* species. The differentiation of *I. domestica* and the other examined European *Iris* taxa does not seem to be adequate if the peak 32, tentatively identified as irisflorentin, is used on its own. Other potential marker of the differentiation – resveratrol, which is possibly present in all of the examined taxa and therefore, offers no selectivity. Although the class of the compounds in the extracts are proposed, further analysis and the use of the certified reference substances are needed in order to provide reliable markers of the species differentiation that could be routinely used in quality control of the plant material.

The largest total phenolics content was found for the taxon *I. pallida*. Both *I. illyrica* and *I. pallida* were the most effective in the antioxidant assays, while the taxa *I. croatica*

and *I. germanica* var. *florentina* were the most effective in the metal chelating assay. Observed differences are the consequence of the variable hydroxylation and methoxylation patterns of Iridaceae type isoflavonoids and differences in the content of other phenolic substances in the extracts, such as xanthones, stilbenes, acetophenones and benzophenones. Compared to the taxon *I. domestica*, which is the most commonly used in phytotherapy, European taxa tend to have superior or similar antioxidant activity. The fact that the taxa were previously widely used for similar indications, together with relatively similar and partly shared chemical profile, might be a sign of the equivalence of the medicinal and wild endemic *Iris* taxa.

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