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Comparative Analysis of Phenolic Compounds in Seven *Hypericum* Species and Their Antioxidant Properties

Gordana Zdunic[a,*], Dejan Godjevac[b], Katarina Savikin[a] and Silvana Petrovic[c]

[a]*Institute for Medicinal Plant Research „Dr Josif Pančić“, Tadeuša Košćuška 1, 11000 Belgrade, Serbia*
[b]*Institute of Chemistry, Technology and Metallurgy, Njegoševa 12, 11000, Belgrade, Serbia*
[c]*Institute of Pharmacognosy, Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*

gzdunic@mocbilja.rs

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A comparative analysis of the phenolic compounds in the 70% EtOH extracts of *Hypericum acutum*, *H. androsaemum*, *H. barbatum*, *H. hirsutum*, *H. maculatum*, and *H. richeri* has been carried out using high-performance liquid chromatography coupled with photodiode array UV detection and high resolution TOF mass spectrometry. Quercetin, astilbin, I3,II8-biapigenin, orientin, 2''-*O*-acetylorientin, three phenolcarboxylic acids, and eight flavonols 3-*O*-glycosides were identified in the extracts on the basis of their on-line UV spectra, accurate mass spectral data, and in comparison of retention times with those from the standards. Fingerprint analysis of the extracts revealed significant differences in the qualitative and quantitative chemical composition of the studied species. Antioxidant assays with various reaction mechanisms were used including ferric reducing antioxidant power (FRAP) assay, DPPH, ABTS, superoxide anion radical scavenging capacity and inhibition of liposome peroxidation induced by Fe^{2+}. The most potent were extracts of *H. acutum* and *H. maculatum* indicating this *Hypericum* species interesting for further research aimed as a potentially new source of biologically active compounds.

Keywords: *Hypericum* species, Chemical analysis, LC/MS, HPLC/DAD, Antioxidant activity.

Hypericum perforatum (St. John's wort) is the only *Hypericum* species officially accepted for its positive pharmacological activities and described in relevant monographs of the European Medicines Agency, European Scientific Association for Phytotherapy, World Health Organization [1-4]. Flowering aerial parts of St. John's wort have been commonly used in a form of tea, dry and liquid hydro alcoholic extracts or as oil extracts. Only standardized, dry hydro alcoholic extracts are used for herbal drug production (oral solid dosage forms) that could be used in the treatment of mild to moderate depressive episode [2]. Furthermore, St. John's wort has been traditionally used for the treatment of mild gastrointestinal complaints, or externally as an antiinflammatory or wound healing agent. Chemical composition of *H. perforatum* has been thoroughly studied and the several groups of compounds are considered as the active principles (naphthodianthrone derivatives hypericin and pseudohypericin, phloroglucinol derivatives hyperforin and adhyperforin, and several flavonoids) [5]. Beside *H. perforatum*, other *Hypericum* species have been traditionally used in ethnomedicine in different countries. Therefore, there is an increasing interest in studying other *Hypericum* species, in order to collect scientific data which could indicate them as a potentially new source of biologically active compounds and to verify its traditional usage. Some of them have been studied regarding their chemical composition and various biological activities (antioxidant, antimicrobial, antidepressive, cytotoxic, antiinflammatory activities) [6-11].

In the present study comparative qualitative and quantitative chemical analysis of the hydroalcoholic extracts of seven *Hypericum* species from Serbia and Montenegro (*H. richeri* Vill., *H. barbatum* Jacq., *H. hirsutum* L., *H. androsaemum* L., *H. acutum* Mnch., *H. maculatum* Crantz, and *H. perforatum* L.) have been performed and its antioxidant effect was evaluated by different *in vitro* methods.

Detailed LC-UV-MS qualitative and HPLC-UV quantitative chemical analysis of the hydroethanolic extracts of *H. richeri, H. barbatum, H. hirsutum, H. androsaemum, H. maculatum* and *H. perforatum* was performed. Identification of the compounds was based on the characteristic UV spectra and molecular formula calculated from precise molecular weight of quasi-molecular ions in ESI mass spectra. Deviations of the calculated weight were below 2.5 ppm for all measurements (Table 1). The final identification of compounds was performed by comparing their retention times, UV and MS spectra with the appropriate retention times and spectra of standard commercially available compounds or flavonoids previously isolated in our laboratory from the *H. richeri* [12]. In the analyzed extracts 17 compounds were identified: (**1**) 3-*O*-, (**2**) 4-*O*-, and (**3**) 5-*O*-caffeoylquinic acid, (**4**) orientin, (**5**) myricetin 3-*O*-rutinoside, (**6**) myricetin 3-*O*-galactoside, (**7**) myricetin 3-*O*-glucoside, (**8**) rutin, (**9**) hyperoside, (**10**) 2''-*O*-acetyl-orientin, (**11**) isoquercitrin, (**12**) quercitrin, (**13**) astilbin, (**14**) quercetin, (**15**) I3,II8-biapigenin, (**16**) pseudohypericin, and (**17**) hypericin.

Comparative analysis of the extracts of selected *Hypericum* species pointed to significant differences in qualitative and quantitative chemical composition (Tables 1 and 2). Of the three identified caffeoylquinic acid, 3-*O*- isomer was not present only in the extract of *H. barbatum*, 5-*O*-isomer only in the extract of *H. maculatum*, while the 4-*O*-caffeoylquinic acid was detected only in the extract of *H. barbatum*. Orientin and its 2''-*O*-acetyl derivative were detected only in the extract of *H. hirsutum*, all three myricetin glycosides only in the extract of *H. richeri*, and astilbin only in the extract of *H. acutum*. The presence of quercetin and its glycosides, in a different qualitative and quantitative ratio is characteristic for all studied species. Hyperoside, isoquercitrin and quercetin were detected in all extracts. Rutin was present only in the extracts of *H. richeri, H. perforatum* and *H. hirsutum.* Quercitrin was not identified only in the extracts of *H. hirsutum* and *H. androsaemum*, and I3,II8-biapigenin only in the extract of *H. androsaemum*.

Table 1: LC/MS data of hydroalcoholic extracts of the studied *Hypericum* species.

	Compound	Rt (min)	Positive mode[a]			Negative mode[b]		Molecular formula	Plant species[c]
			Measured mass		Mass error	Measured mass	Mass error		
1	3-*O*-Caffeoylquinic acid	9.2	354.0951	M	0.0	354.0959	2.4	$C_{16}H_{18}O_9$	Ac, An, H, M, R, P
			162.0315	M-Caff[d]	1.1			$C_9H_6O_3$	
2	4-*O*-Caffeoylquinic acid	12.8	354.0953	M	0.6	354.095	0.1	$C_{16}H_{18}O_9$	B
			162.0314	M-Caff	1.7			$C_9H_6O_3$	
3	5-*O*-Caffeoylquinic acid	16.1	354.0952	M	0.4	354.095	0.1	$C_{16}H_{18}O_9$	Ac, An, H, B, R, P
			162.0315	M-Caff	1.1			$C_9H_6O_3$	
4	Orientin	22.9	448.1008	M	0.4	448.1007	0.7	$C_{21}H_{20}O_{11}$	H
5	Myricetin 3-*O*-rutinoside	23.3	626.1491	M	1.3	626.1485	0.4	$C_{27}H_{30}O_{17}$	R
			480.0906	M-Rha	0.5			$C_{21}H_{20}O_{13}$	
			318.0373	M-Rha-Hex	0.9			$C_{15}H_{10}O_8$	
6	Myricetin 3-*O*-galactoside	23.9	480.0906	M	0.5	480.0911	1.5	$C_{21}H_{20}O_{13}$	R
			318.0373	M-Hex	0.9			$C_{15}H_{10}O_8$	
7	Myricetin 3-*O*-glucoside	24.6	480.0910	M	1.4	480.0909	1.1	$C_{21}H_{20}O_{13}$	R
			318.0379	M-Hex	1.1			$C_{15}H_{10}O_8$	
8	Rutin	31.2	610.1528	M	0.9	610.1535	0.2	$C_{27}H_{30}O_{16}$	H, R, P
			464.0957	M-Rha	0.5			$C_{21}H_{20}O_{12}$	
			432.1059	M-Hex	0.7			$C_{21}H_{20}O_{10}$	
			302.0427	M-Rha-Hex	0.0			$C_{15}H_{10}O_7$	
9	Hyperoside	33.0	464.0956	M	0.2	464.0957	0.7	$C_{21}H_{20}O_{12}$	Ac, An, H, B, M, R, P
			302.0426	M-Hex	0.4			$C_{15}H_{10}O_7$	
10	2''-O-Acetyl-orientin	34.3	490.1118	M	1.4	490.1114	0.6	$C_{23}H_{22}O_{12}$	H
11	Isoquercitrin	34.5	464.0958	M	0.5	464.0957	0.7	$C_{21}H_{20}O_{12}$	Ac, An, H, B, M, R, P
			302.0425	M-Hex	0.8			$C_{15}H_{10}O_7$	
12	Quercitrin	36.9	448.1001	M	0.9	448.101	1.0	$C_{21}H_{20}O_{10}$	Ac, B, M, R, P
13	Astilbin	37.8	450.1161	M	0.2	450.1162	0.0	$C_{21}H_{22}O_{11}$	Ac
			276.0632	M-Rha	0.6			$C_{14}H_{12}O_6$	
14	Quercetin	40.1	302.0431	M	1.5	302.0421	2.0	$C_{15}H_{10}O_7$	Ac, An, H, B, M, R, P
15	I3,II8-Biapigenin	42.2	538.0894	M	1.1	538.0893	1.3	$C_{30}H_{18}O_{10}$	Ac, H, B, M, R, P

[a] Mass calculated from ions e.g. M+H. M+Na. M+K, and 2M+H.
[b] Mass calculated from M-H ions.
[c] R - *H. richeri*,P - *H. perforatum*, B - *H. barbatum*, H - *H. hirsutum*, An - *H. androsaemum*, Ac - *H. acutum*, M - *H. maculatum*.
[d] Caff - caffeoyl, Hex - hexosyl, Rha – rhamnosyl.

Table 2: Content of phenolic compounds in hydroalcoholic extracts of the studied *Hypericum* species.

	Compound	λ (nm)	(mg/g dw)[a] *H. richeri*	(mg/g dw) *H. perforatum*	(mg/g dw) *H. barbatum*	(mg/g dw) *H. hirsutum*	(mg/g dw) *H. androsaemum*	(mg/g dw) *H. acutum*	(mg/g dw) *H. maculatum*
1	3-*O*-Caffeoylquinic acid	320	21.5 ± 0.2	3.8 ± 0.1	n.d.	6.8 ± 0.1	28.2 ± 0.2	10.3 ± 0.1	7.1 ± 0.1
2	4-*O*-Caffeoylquinic acid	320	n.d.[b]	n.d.	0.5 ± 0.2	n.d.	n.d.	n.d.	n.d.
3	5-*O*-Caffeoylquinic acid	320	3.0 ± 0.1	5.1 ± 0.1	30.7 ± 0.2	1.0 ± 0.3	25.4 ± 0.2	1.0 ± 0.3	n.d.
4	Orientin	254	n.d.	n.d.	n.d.	29.6 ± 0.3	n.d.	n.d.	n.d.
5	Myricetin 3-*O*-rutinoside	254	63.1 ± 1.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	Myricetin 3-*O*-galactoside	254	25.9 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	Myricetin 3-*O*-glucoside	254	33.2 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	Rutin	254	61.0 ± 1.6	17.0 ± 0.5	n.d.	1.5 ± 0.2	n.d.	n.d.	n.d.
9	Hyperoside	254	15.7 ± 0.2	15.5 ± 0.2	33.7 ± 0.2	2.4 ± 0.2	1.8 ± 0.2	16.4 ± 0.1	6.1 ± 0.1
10	2''-*O*-Acetyl-orientin	254	n.d.	n.d.	n.d.	38.5 ± 0.3	n.d.	n.d.	n.d.
11	Isoquercitrin	254	14.5 ± 0.4	7.2 ± 0.1	0.4 ± 0.1	10.5 ± 0.2	0.3 ± 0.1	8.4 ± 0.1	1.2 ± 0.1
12	Quercitrin	254	7.1 ± 0.2	3.9 ± 0.1	9.1 ± 0.1	n.d.	n.d.	0.4 ± 0.2	0.15 ± 0.05
14	Quercetin	254	3.2 ± 0.1	5.8 ± 0.1	0.3 ± 0.1	3.9 ± 0.1	1.1 ± 0.2	7.8 ± 0.1	8.0 ± 0.1
15	I3,II8-Biapigenin	320	3.0 ± 0.1	2.9 ± 0.1	0.4 ± 0.1	4.1 ± 0.1	n.d.	0.5 ± 0.1	0.3 ± 0.1
16	Pseudohypericin	590	4.5 ± 0.1	4.2 ± 0.1	5.0 ± 0.2	n.d.	n.d.	0.88 ± 0.02	1.5 ± 0.1
17	Hypericin	590	0.4 ± 0.1	2.0 ± 0.1	3.8 ± 0.1	0.82 ± 0.03	n.d.	0.62 ± 0.02	1.1 ± 0.1
	Total flavonoid content		226.7 ± 4.2	52.3 ± 0.9	43.9 ± 0.5	90.5 ± 1.1	3.2 ± 0.3	33.5 ± 0.5	15.8 ± 0.4

[a] Values are expressed as mean ± standard deviation.
[b] Not detected.

Naphthodianthrones were not identified in the extract of *H. androsaemum*, which is consistent with findings in the literature [13,14]. In the extract of *H. hirsutum* only hypericin was identified as it was stated previously [14,15]. In all other analyzed extracts hypericin and pseudohypericin were also present.

The presence of 3-*O*-caffeoylquinic acid, rutin, hyperoside, quercitrin, isoquercitrin, quercetin, hypericin and pseudohypericin in *H. richeri* was confirmed in previous studies [13,16,17], while 5-*O*-caffeoylquinic acid, myricetin 3-*O*-rutinoside, myricetin 3-*O*-galactoside, myricetin 3-*O*-glucoside and I3,II8-biapigenin were detected for the first time in this species. Polyphenolic composition of the species *H. perforatum* is in accordance with findings in the literature [18-20]. According to the literature, in the methanol extract of *H. barbatum* hyperoside, quercitrin, quercetin, isoquercitrin, hyperforin, hypericin and pseudohypericin have been detected so far [13,21]; I3, II8-biapigenin, as well as 4-*O*- and 5-*O*-caffeoylquinic acid were identified in *H. barbatum* for the first time in our study. Polyphenolic compounds 3-*O*-caffeoylquinic acid, rutin, hyperoside, quercitrin, isoquercitrin, quercetin, orientin, 2'-*O*-acetyl-orientin, pseudohypericin and hypericin were previously detected in the *H. hirsutum* [22-25], while 5-*O*-caffeoylquinic acid was detected for the first time in this species. In previous studies in *H. androsaemum* 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, hyperoside, quercitrin, isoquercitrin, kaempferol, quercetin, quercetin 3-*O*-arabinoside and quercetin 3-sulphate were detected [23,26]. In our extract 3-*O*- and 5-*O*-caffeoylquinic acid, hyperoside, quercitrin, isoquercitrin, and quercetin were also identified. The presence of 3-*O*-caffeoylquinic acid, hyperoside, quercitrin, isoquercitrin, quercetin, I3,II8-biapigenin,

pseudohypericin and hypericin in *H. maculatum* is consistent with the results of other authors [13,24,27,28]. The chemical composition of species *H. acutum* has not been thoroughly studied so far. Only by using thin layer chromatography rutin, hyperoside, quercitrin, quercetin, caffeic and 3-*O*-caffeoylquinic acid were detected [29]. According to the LC-UV-MS analysis, apart from hyperoside, quercitrin, quercetin and 3-*O*-caffeoylquinic acid, there were for the first time identified also 5-*O*-caffeoylquinic acid, isoquercitrin, astilbin, I3,II8-biapigenin, pseudohypericin and hypericin.

According to the results of quantitative analysis, the most abundant in flavonoids was the extract of *H. richeri,* where myricetin 3-*O*-rutinosid and rutin were the most dominant. Baroni Fornasiero *et al*. have been previously determined contents of hyperoside, isoquercitrin, quercitrin, quercetin, hypericin and pseudohypericin in methanol-acetone (1:1) extract by HPTLC technique, where hyperoside was the most dominant [30].

The second best in flavonoid content was extract of *H. hirsutum*, with the largest share of orientin and its 2'-*O*-acetyl derivative. Although these two flavonoids were previously detected in this species [25], in the literature there is no data on their content. According to the literature, hyperoside was the most common compound in various types of extracts of *H. hirsutum* [22-24].

In *H. barbatum* and *H. acutum* extracts hyperoside was the most dominant, in *H. perforatum* the most dominant were rutin and hyperoside, and in *H. maculatum* quercetin and hyperoside. It was reported earlier that the compound hyperoside is dominant in species *H. barbatum* [13] and *H. maculatum* [13,24,28]. Previous research by other authors determined that the content of certain compounds in *H. perforatum* may vary depending on the period of harvest and environmental factors. Thus, for example, it was found that the content of rutin is largest in the process of formation of flower buds (budding), while the content of quercitrin, quercetin and I3,II8 biapigenin is the largest in the stage of full flowering of the St John's wort [18-20].

The extract of *H. androsaemum* was set out by the lowest flavonoid content, as well as the largest content of phenol-carboxylic acids. Valentao *et al*. have previously shown that there were variations in the content of these phenolic compounds in different types of samples of *H. androsaemum*, which they explained by the possible existence of chemical polymorphism [26]. The quantitative composition of *H. androsaemum* was researched by Šmelcerović and associates, who have managed to determine only the content of hyperforin and hyperoside in this species by LC-MS-MS method [23].

The content of naphthodianthrones was the highest in the extracts of *H. barbatum, H. perforatum* and *H. richeri.* In the extracts of *H. richeri*, *H. perforatum*, *H. barbatum*, *H. acutum* and *H. maculatum*, where both naphthodianthrones were detected, pseudohypericin was more abundant than hypericin, which was most pronounced in the extract of *H. richeri*. Determined higher content of pseudohypericin compared to hypericin in selected *Hypericum* extracts is in accordance with the results obtained previously by Kitanov [15].

The imbalance between the reactive oxygen species and antioxidant defense mechanisms can lead to damage of the cellular membranes, or intracellular molecules, resulting in lipid peroxides that could damage nucleic acids, carbohydrates, proteins and fats. Oxidative stress can be associated with aging, development of atherosclerosis, cancer and neurodegenerative diseases such as Parkinson's and Alzheimer's disease [31,32]. Flavonoids and phenolcarboxylic acid, as the most common species of plant phenolic compounds, are the main carriers of the antioxidant action of the majority of plant species. Their antioxidant activity is well documented in various *in vitro* and *in vivo* experimental models [33-44].

The antioxidant activity of the selected *Hypericum* extracts measured by FRAP test was in the range of 3.6 – 4.5 μmol Fe^{2+}/mg dw (Table 3). The highest antioxidant activity was measured for *H. acutum* (4.5 μmol Fe^{2+}/mg) and *H. maculatum* (4.1 μmol Fe^{2+}/mg) extracts, whereas the *H. barbatum* (3.6 μmol Fe^{2+}/mg) and *H. perforatum* (3.7 μmol Fe^{2+}/mg) extracts showed the weakest activity. According to available literature data, the ability of the reduction of the complex Fe^{3+}-tripyridyltriazine [45] was previously investigated for methanolic extracts of *H. perforatum*, *H. richeri*, *H. barbatum* and *H. maculatum*, obtained after extraction of the plant material with methylene chloride. Similar to our results, *H. maculatum* extract has shown the highest activity measured by FRAP assay, and the least active were extracts of *H. barbatum* and *H. richeri* [6].

The highest DPPH activity have shown extracts of *H. maculatum* (EC_{50} = 10.2 μg/mL) and *H. acutum* (EC_{50} = 10.9 μg/mL), following by *H. hirsutum* and *H. androsaemum* extracts, while the least active were *H. perforatum*, *H. richeri* and *H. barbatum* extracts (EC_{50} = 20.5 μg/mL, EC_{50} = 20.7 μg/mL and EC_{50} = 21.3 μg/mL, respectively). All tested *Hypericum* extracts showed stronger DPPH activity compared to the activity of lyophilized water extract of green tea (EC_{50} = 44.3μg/mL) obtained by extraction of 5 g of green tea with 200 mL of water, 20 min at 80ºC [46]. The obtained results are showing significant ability for neutralization of DPPH radicals of all tested extracts which are consistent with existing literature data. For 80% ethanol extract of *H. perforatum* EC_{50} was 21 μg/mL[47], while the EC_{50} values ranged from 9,0 to 11,7μg/mL for lyophilized water extracts (10 g of plant material of various origin was extracted with 100 mL of water for 10 min at 100ºC) [48]. The acetone-methanol extracts of the flowering aerial parts of *H. perforatum* collected from different locations, neutralized 27 to 36% of DPPH radicals at maximum tested concentration of 12 mg/mL [22]. A similar activity was shown by the extract of *H. hirsutum* (31%) prepared by the same method. Dry methanolic extract of *H. barbatum* obtained after pre-extraction of the plant material with methylene chloride, neutralized 31.9% of the DPPH radicals at a concentration of 10 mg/mL; more active was the extract of *H. richeri* (49.4%), while an even greater ability to neutralize DPPH radicals was shown by the extract of *H. maculatum* (56.2%) [6]. Almeida *et al*. have also shown a high ability of lyophilized 40% ethanolic extract of *H. androsaemum* to neutralize the DPPH radical (EC_{50} = 11.3 mg/mL) [49].

Within the $ABTS^{\bullet+}$ test, the antioxidant capacity of the tested extracts was expressed as a TEAC value (Trolox Equivalent Antioxidant Capacity) as mmol Trolox/g of dry extract. Of all the tested samples, the extract of *H. acutum* exhibited the highest antioxidant capacity with the TEAC value of 1.69 mmol/g Trolox, whereas the extracts of *H. barbatum* and *H. richeri* (0.64 mmol Trolox/g and 0.65 mmol Trolox/g) showed the weakest activity (Table 3). The activity of 1 g of *H. acutum* extract is equivalent to the activity exhibited by the 85 mL of green tea extract (1.5 g of green tea were extracted with 180 mL water for 5 minutes at 80°C) [50]. According to the results by Zheleva-Dimitrova *et al*, methanol extracts of *H. barbatum*, obtained after prior extraction with methylene chloride, at a concentration of 10 mg/mL, have also shown the lowest ability of neutralization of ABTS radicals compared to the activity of the *H. perforatum*, *H. maculatum* and *H. richeri* extracts [6].

Table 3: Antioxidant properties of hydroalcoholic extracts of the studied *Hypericum* species.

Extract	FRAP[a] (μmol Fe^{2+}/mg)	DPPH[b] EC_{50} (μg/mL)	TEAC[c] (mmol Trolox/g dw)	SO[d] EC_{50} (μg/mL)	LP[e] EC_{50} (μg/mL)
H. richeri	4.1 ± 0.2	20.7 ± 0.1	0.65 ± 0.07	249 ± 8	56 ± 3
H. perforatum	3.7 ± 0.1	20.5 ± 0.1	1.02 ± 0.06	n.d.	53 ± 3
H. barbatum	3.6 ± 0.1	21.3 ± 0.1	0.64 ± 0.02	n.d.	n.d.
H. hirsutum	3.9 ± 0.2	13.6 ± 0.1	1.06 ± 0.08	183 ± 6	48 ± 3
H. androsaemum	4.0 ± 0.2	15.4 ± 0.1	1.36 ± 0.06	125 ± 4	41 ± 3
H. acutum	4.5 ± 0.2	10.9 ± 0.1	1.69 ± 0.09	177 ± 5	26 ± 2
H. maculatum	4.1 ± 0.2	10.2 ± 0.1	0.74 ± 0.06	243 ± 8	33 ± 2
Trolox	/	6.1 ± 0.2	/	54 ± 3	4.5 ± 0.1

[a]Ferric Reducing Ability of Plasma; [b]2,2-diphenyl-1-picrylhydrazyl; [c]Trolox equivalent antioxidant capacity;[d]Superoxide radical anion scavenging assay; [e]Lipid peroxidation assay; n.d.- with max tested concentrations 50% of inhibition wasn't achieved.

In contrast to the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, superoxide anion radical is a reactive oxygen species that can be found in biological systems. From the superoxide radical through Fenton reaction more reactive oxygen species, such as singlet oxygen, hydroxyl radical and hydrogen peroxide are easily formed and they could easily initiate lipid peroxidation [51], and damage carbohydrates, proteins and DNA [52]. The ability of the tested extracts to neutralize superoxide anion radical was measured by the concentration of the extracts that neutralize 50% of the radicals (EC_{50} values) created during transfer of electrons from NADH to the oxygen present in the aqueous system. The highest activity have shown extracts of *H. androsaemum* (EC_{50} = 125 μg/mL) and *H. acutum* (EC_{50} = 177 μg/mL), followed by the extracts of the species *H. hirsutum*, *H. maculatum* and *H. richeri* (Table 3). Extracts of the *H. perforatum* and *H. barbatum* with maximum tested concentrations did not reach EC_{50}. The high ability of neutralization of superoxide anion radical of *H. androsaemum*, that was in the same level as the lyophilized water extract of green tea EC_{50} = 164,8 μg/mL [46], which was confirmed by other authors too. According to the results by Almeida *et al*, for lyophilized 40% ethanol extract EC_{50} was 32.7 mg/mL [49], while for lyophilized aqueous extract obtained by boiling EC_{50} was 25.6 mg/mL [53]. Zou *et al*. have examined the ability of neutralizing superoxide anion radical for a commercial extract of *H. perforatum*, which in a subsequent purification on a non-ionic resin column was enriched in flavonoids, and showed moderate antiradical activity in this test [54]. Also, this type of activity was researched by Orčić *et al*. for methanol extract fractions of *H. perforatum.* The fractions in which the highest content of some biflavonoids, naphthodianthrones and phloroglucinols, as well as the lowest content of flavonoids and phenol-carboxylic acids was determined, exhibited the weakest ability to neutralize superoxide anion radicals [45].

In biological systems, the lipid peroxidation involves the oxidative degradation of polyunsaturated fatty acids in cell membranes, where the key role is played by a hydroxyl radical which in the *in vivo* environment is mainly generated as a product of the so-called Fenton's reaction, where the hydroxyl radical is formed from H_2O_2 in the presence of ferric salts [55].The resulting lipid radical in reaction with oxygen provides the lipid peroxyl radical which with a new molecule of the polyunsaturated fatty acids builds a lipid peroxide. In our study, the ability to inhibit lipid peroxidation of the liposomes was determined spectrophotometrically by measuring the intensity of pink TBA complexes and aldehydes generated by decomposition of lipid peroxides at 532 nm. The oxidation of the liposome was caused by the redox system of the ascorbic acid/$FeCl_3$ in which by the Fenton reaction hydroxyl radicals was generated. Testing of the ability of inhibiting lipid peroxidation has an advantage compared to the tests with synthetic stable nitrogen radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ do not have great similarities with the highly reactive superoxide radical [56].

Of the tested *Hypericum* extracts, extracts of the *H. acutum* and *H. maculatum* have shown the greatest ability to inhibit lipid peroxidation (EC_{50} = 26 μg/mL and EC_{50} = 33 μg/mL, respectively) (Table 3). All extracts showed lower activity compared to the lyophilized green tea aqueous extract (5 g of drug extracted with 200 mL of water, for 20 min at 80°C) whose EC_{50} value was 1.94 mg / mL [46]. Silva et al. have investigated the ability to inhibit lipid peroxidation in isolated rat synaptosomes, and demonstrated that the 80% ethanol extract of *H. perforatum* (EC_{50} = 27.7 μg/mL) results in a significant inhibition of lipid peroxidation [57]. According to available literature data, the ability of *H. richeri*, *H. barbatum* and *H. maculatum* to inhibit lipid peroxidation was investigated by thiocyanate test based on the inhibition of peroxidation of linoleic acid [6]. Based on these results, although they were slightly lower compared to the synthetic antioxidant BHT, it was found that the methanol extracts of these species, obtained after extraction with methylene chloride, possess significant antioxidant activity. For the extract of *H. hirsutum* (methanol:acetone=1:1) the antioxidant activity was tested in the system of β-carotene/linolenic acid, wherein 0.1% of tested extract reached the percentage of inhibition of lipid peroxidation of 35% [22].

Hydroalcoholic extracts of *H. acutum* and *H. maculatum* possessed the strongest antioxidant potential according to the results of performed tests (FRAP assay, DPPH radicals neutralization capacity and the inhibition of lipid peroxidation), while the lowest activity showed extracts of *H. barbatum, H. richeri* and *H. perforatum*. The resulting activity of the extracts can be explained by differences in their chemical composition. In the case of ABTS$^{\bullet+}$ test and the ability to neutralize superoxide radicals, most active were the extracts of *H. acutum* and *H. androsaemum*, while the lowest activity in these tests was also demonstrated by the extracts of *H. barbatum, H. richeri* and *H. perforatum.*

According to the results by Orčić *et al*., the fractions of the methanol extract of *H. perforatum*, with higher content of flavonoids and phenol-carboxylic acids, demonstrated the superior activity compared to the synthetic antioxidant BHT in the FRAP test, tests of neutralization superoxide anion and DPPH radical as well as in the lipid peroxidation inhibition test. Low activity in all the tests (with the exception of lipid peroxidation) was shown by the fractions in which dominated naphthodianthrones and biflavonoids [45]. Despite the weak antiradical activity, biflavonoids in the test of inhibition of lipid peroxidation have shown a high antioxidant activity, in contrast to naphthodianthrones which in this test also did not show any antioxidant activity [57]. Cai *et al* have studied the antioxidant activity of a large number of different classes of compounds, including the activity of phenolic acids, flavonoids, tannins, using ABTS and DPPH assay. In the group of flavonoids, the highest antioxidant activity showed quercetin, followed by epigallocatechin-gallate and epicatechin gallate, which are the dominant components of tea. TEAC value for quercetin was 4.42

mM, and it was approximately two times higher compared to the TEAC values for its glycosides [58]. Quercetin belongs to the class of flavonoids, which in addition to the hydroxyl group in position C-3, includes 3',4'-dihydroxy groups in B ring and 2,3-double bond is conjugated with the carbonyl group in C-4 position. These structural characteristics represent the basis for high free radicals neutralization capability [33,59]. Glycosylation of 3-OH group leads to a significant reduction in the antioxidant capacity of these molecules [33]. High antioxidant activity of the *H. acutum* and *H. maculatum* extracts could be explained at least partially by high quercetin content. On the other side, the extract of *H. barbatum* which had the lowest content of quercetin showed the weakest antioxidant activity as well. Cai *et al*. have also found that myricetin shows a significantly weaker radical neutralizing ability (TEAC value of 1.31 mM) in relation to the quercetin, despite the fact that flavonol myricetin has six hydroxyl groups and *ortho*-dihydroxy structure [58]. That could be a possible explanation why *H. richeri* extract, even though it is abundant in three myricetin glycosides, showed weak antioxidant activity. In this assay, 3-*O*-caffeoylquinic acid showed similar activity (TEAC value of 1.56 mM) [58]. The ability of flavonoids to inhibit lipid peroxidation investigated Silva *et al*. where quercetin also showed the highest antioxidant activity (EC_{50} = 0.08 uM), while its glycosides (rutin, hyperoside, quercitrin and isoquercitrin) and I3, II8-biapigenin showed less activity [57].

According to the results of performed qualitative and quantitative chemical analysis and shown antioxidant properties of tested *Hypericum* species, *H. acutum* and *H. maculatum*are the most interesting species for further research as potentially new sources of biologically active compounds.

Experimental

Plant material: The flowering aerial parts of *H. richeri* were collected in July 2005 on Bogićevica mountain, Montenegro; *H. perforatum* on Suvobor mountain, Serbia in 2007; *H. barbatum* on Suvobor mountain in 2007; *H. hirsutum* on Povlen mountain, Serbia in 2007; *H. androsaemum* on mountain Maljen, Serbia in 2007; *H. acutum* and *H. maculatum* on mountain Jablanik, Serbia in 2007. The voucher specimens (No. B314/05, S286/07, S287/07, P35/07, M36/07, J37/07, J38/07, respectively) were deposited in the Biology Department, Faculty of Science, the University of Montenegro, Podgorica. Air-dried, grounded plant material was macerated with 70% EtOH at room temperature for 72h. After filtration, solvent was evaporated under vacuum at temperature bellow 40°C. Dry extracts were kept in vacuum desiccator until further analysis.

Qualitative chemical analysis: For LC/MS analysis Agilent MSD TOF coupled to an Agilent 1200 series HPLC was used. Dry extracts were dissolved and filtered through a 0.45 □m filter prior injection. Analyses were carried out on a column LiChrospher RP-18e (5 µm, 250×4 mm I.D.) (Merck); flow rate 1 mL/min; mobile phase: A (99% H_2O, 1% formic acid), B (Acetonitrile); elution with gradient program (0-12 min 8-10% B, 12-14 min 10-16% B, 14-30 min 16% B, 30-36 min 16-36% B, 36-42 min 36-60% B, 42-46 min 60-100% B), at flow rate 1 mL/min, injection volume 7 μl. Detection: 254 nm, 320 nm, and 590 nm. For the MS, mass spectra were acquired using an Agilent ESI-MSD TOF. Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psig; drying gas temperature was 350°C; using polarity switching mode. For positive ESI analysis, the parameters were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V. For negative ESI analysis, the parameters were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V, 250 V. The mass range was from 100 to 1500 *m*/*z*. The data acquire was at one scan per second; with each scan composed of 10000 transients. Data acquisition and processing was done with the software Molecular Feature Extractor.

Quantitative chemical analysis: HPLC/DAD analysis of the extracts was performed on a Hewlett Packard HPLC model 1200; DAD detector (HP 1040); the column and operating conditions were same as those for LC/MS analysis, except mobile phase A (0.1% formic acid was used instead of 0.1% phosphoric acid). Concentrations of the commercial standards used for the calibration were: 0.2-2.0 mg/mL for flavonoids, 0.1-1.0 mg/mL for naphthodianthrones, 0.1-0.5 mg/mL for caffeoylquinic acids. For myricetin 3-*O*-rutinoside, myricetin 3-*O*- glucopyranoside, and myricetin 3-*O*-galactopyranoside commercial standards were not available, and they were assayed as myricetin equivalents.

FRAP assay: Assessment of total antioxidant capacity of *Hypericum* extracts was performed by FRAP assay (Ferric Reducing Ability of Plasma) [60]. FRAP reagent was prepared by adding 2.5 mL of a TPTZ solution (10 mmol/l of 2,4,6-tripyridyl-s-triazine in 40 mmol/l of hydrochloric acid) and 2.5 mL of a solution of $FeCl_3$ x 6 H_2O (20 mmol/l in distilled water) in 25 mL of acetate buffer (300 mmol/l, pH = 3.6). In the test tube 3 mL of FRAP reagent was mixed with 100 mL of the appropriate concentration of the extract dissolved in methanol and incubated for 30 min at 37°C. The absorbance of the solution was measured at 593 nm. For calibration different concentrations of $FeSO_4$ aqueous solutions (in the range of 100-1000 mmol/l) were used. The total antioxidant capacity of the extracts was expressed as Fe^{2+}µmol/ mg. The obtained results represent the mean of three determinations.

DPPH assay: The ability of the *Hypericum* extracts to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was conducted according to the procedure described previously [61]. A series of properly diluted extracts in 70% ethanol (4 mL) was mixed with 1 mL of a 0.5 mM solution of DPPH in 70% ethanol and left in the dark for 30 min. The absorbance of the solution was measured at 517 nm. The percentage of neutralization of DPPH radicals was calculated using the following formula: I (%) = [(Ac-Aa)/Ac] × 100; where Ac is the absorbance of the negative control (solvent), and Aa is the absorbance of the samples. EC_{50} values were estimated using a nonlinear regression algorithm. All test analyses were run in triplicate.

$ABTS^{\bullet+}$ scavenging assay: The ability of the extracts to scavenge $ABTS^{\bullet+}$ was measured as described by Re *et al*. [25] with slight modifications. The $ABTS^{\bullet+}$ was generated by reacting 7 mM aqueous ABTS with 2.45 mM $K_2S_2O_8$ in the dark at room temperature for 16 h. For the measurement of scavenging capacity, solution of $ABTS^{\bullet+}$ was diluted with MeOH to an absorbance of 0.7 at 734 nm. Solutions of samples in MeOH were prepared so that, after adding of a 100 μL aliquot of each dilution into the assay, they produced between 10-80% inhibitions of the absorbance comparing to those of control. After addition of 900 μL of diluted $ABTS^{\bullet+}$ solution to 100 μL of the sample, the absorbance at 734 nm was continuously recorded for 5 min. The scavenging percentage was calculated using the following equation: $S = [(A_c-A_s)/A_c] \times 100$; where S was the scavenging percentage, A_c was the absorbance of the negative control (contained 100 μL of MeOH), and A_s was the absorbance of the samples. The scavenging percentage was plotted as a function of concentration of the samples. The antioxidant capacity is expressed as Trolox equivalent antioxidant capacity (TEAC) values (mmol Trolox/g dry extract).

Superoxide radical anion scavenging assay: Superoxide radical anion ($O_2^{\bullet -}$) scavenging capacity of the extracts was performed using NADH/PMS system following a procedure described by Valentao *et al*. [62] with some modifications. Briefly, various concentrations of the extracts were prepared in MeOH and mixed with NADH (468 μM), NBT (156 μM), and PMS (60 μM) dissolved in phosphate buffer (100 mM, pH 7.4). The reduction of NBT to the blue chromogen formazan by $O_2^{\bullet -}$ was monitored on spectrophotometer at 560 nm for 5 min. The scavenging percentage was then calculated and plotted as a function of concentration of the samples. EC_{50} values were estimated using a nonlinear regression algorithm. All test analyses were run in triplicate.

Lipid peroxidation assay: The ability of the extract to inhibit phospholipids degradation was determined by modified method of Liu *et al*. [62]. For the preparation of liposomes, a $CHCl_3$ solution of L-α-phosphatidylcholine was evaporated under vacuum to dryness. Buffer solution (10 mM NaH_2PO_4, pH 7.4) was added to obtain final concentration of 10 mg lipid/mL and the suspension was vortexed for 5 min. Liposomes (0.5 mg/mL) were incubated at 37°C for 60 min with the extracts of varying concentrations, Mohr's salt (290 µM) and ascorbic acid (100 µM) in phosphate buffer (70 mM, pH = 7.4), with final volume of 4 mL. The reaction was terminated by adding 1 mL of 20% trichloracetic acid and 1 mL of 1% 2-thiobarbituric acid in 0.05 M KOH, and the solutions were then heated in a water bath at 100°C for 15 min. The suspensions were centrifuged for 15 min at 3000-4000 rpm. Thiobarbituric acid reactive substances gave pink color of supernatant, and the absorbance was measured at 532 nm. The percentage inhibition was calculated, and EC_{50} values were then estimated using a nonlinear regression algorithm. All test analyses were run in triplicate. Trolox was used as a positive control.

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