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THE PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT CAPACITY DETERMINATION OF FIVE *HYPERICUM* SPECIES

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ABSTRACT. Medicinal plants are a valuable source of lead compounds and novel drugs. The *Hypericum* L. species traditionally treats skin wounds, sciatica, and depression. Presently, only a small percentage of the *Hypericum* species have been phytochemically characterised and 60% still requires analysis. Ethanolic and methanolic extracts were obtained by ultrasound assisted extraction and maceration extraction methods. Polyphenols, sterols, methoxyflavones, hyperforin and hypericin were detected through HPLC-MS analysis. ABTS assay was used to evaluate the total antioxidant capacity. *H. maculatum* and *H. moserianum* had the highest antioxidant capacity. The most abundant polyphenol in *H. humifusum, H. moserianum* and *H. miracle-pistache* was chlorogenic acid. For *H. maculatum* and *H. perforatum*, hyperoside and rutoside were the most abundant polyphenols. Sterols and methoxyflavones were quantified for the first time in all the species. β-sitosterol was the most abundant sterol across all species and ergosterol was absent in all species. Hispidulin was the only methoxyflavone (in small concentrations) found in all species except *H. miracle-pistache*. Hypericin was absent in *H. moserianum* and *H. miracle-pistache* and was most abundant in *H. maculatum.* Interestingly, *H. miracle-pistache* had higher concentrations of hyperforin than *H. perforatum.* The phytochemical profile of analysed *Hypericum* species prove to be a valuable bioactive's source.

Keywords: *Phytochemical analysis, antioxidant activity, Hypericum species, hyperforin, hypericin*

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INTRODUCTION

The *Hypericum* L. genus belongs to the *Hypericaceae* family containing 484 species [1]. According to medicinal folklore, the *Hypericum* species can be used both internally and externally with various therapeutic applications such as a diuretic, used to treat sciatica, skin wounds, burns, eczema, and depression [2]. Previous literature has identified flavonoids (e.g. quercetin and hyperoside), phloroglucinol derivates (e.g. hyperforin) and naphthodianthrones (e.g hypericin) as common constituents to the *Hypericum* species [3]. Presently, only a small percentage of the *Hypericum* species have been phytochemically characterized and 60% still require analysis [4]. This propels the need to screen more *Hypericum* species for novel bioactives with pharmacological properties.

H. perforatum (St. John's wort) is the most commercially recognized member of the species, with a range of pharmacological properties [5]. These include antiviral, anti-inflammatory, wound-healing, and apoptosis-inducing properties [6]. Many studies have demonstrated its high efficacy in treating mild to moderate depression with few adverse effects, making it safer compared to other antidepressants [7]. Hyperforin (responsible for the antidepressant activity) and hypericin (causes photosensitivity to the skin) are the two most studied phytochemicals in *H. perforatum.* However, research has concluded that the broad range of pharmacological activities of *H. perforatum* is not dependent on a single compound [8] but is a result of the synergistic efforts of several constituents, which cannot be separated into active compounds [10].

Hyperforin, a natural phloroglucinol [9], is the most neuroprotective bioactive in the plant [10], eminent for its treatment of mild to moderate depression with fewer side effects over other major antidepressants [11]. Hyperforin inhibits serotonin uptake by activating the transient receptor potential channel protein 6 (TRPC6) [12]. This increases the intracellular concentrations of sodium and calcium, resulting in a decreased sodium gradient between the neurons and synapse, which ultimately decreases monoamine neurotransmitter reuptake. This mechanism differs from the conventional antidepressants, possibly pointing to a new class of antidepressants [13]. Hyperforin also displays antitumor, antiangiogenic, and antibacterial activity [16].

Hypericin is a polycyclic quinone [14] with four hydroxyl groups adjacent to two carbonyl groups [15]. The hydroxyl hydrogen can transfer the hydroxyl oxygen and carbonyl oxygen in the presence of fluorescent light due to the resonance of the structure and relatively short distance between the oxygen atoms (~2.5 Å). As a result, when exposed to fluorescent light, the hydrogen is constantly in flux between the two oxygen atoms. This makes hypericin very photoreactive, as it can generate reactive oxygen species and

singlet oxygen ($^1O_2$), acting as a sensitizer in photodynamic reactions (type II mechanism) [16]. This can lead to lipid peroxidation, severe necrosis and sunburn to the skin when ingested excessively by humans [17]. However, the photo-reactivity of hypericin has demonstrated antiviral properties, including the inhibition of protein-kinase activity needed for replication of some viruses [18].

Considering the pharmacological and possible therapeutic uses of hypericin and hyperforin, quantifying their concentrations in each *Hypericum* species were of special interest. Identifying more natural sources of these compounds could be used for more clinical studies.

Lipid peroxidation and free radicals contribute pathogenically to some chronic diseases such as atherosclerosis, coronary heart disease and cancer [19]. The cardioprotective and anticarcinogenic effects of phenolic compounds are attributed to their antioxidant activity which alleviates lipid peroxidation and obviates free radicals [20]. Several epidemiological studies have agreed that exogenous antioxidant intake effectively prevents or suppress such diseases. Therapeutic use of natural antioxidants has gained global interest for preventing oxidative damage. Numerous phytochemicals in Hypericum reportedly act as antioxidants, such as flavonoids and tannins. These have displayed radical scavenging in a dose-dependent manner, showing potential for therapeutic drug use for conditions associated with free radical pathology [21].

Very few phytochemical and antioxidant reports exist for *H. humifusum, H. maculatum* and *H. moserianum* and no previous study exist for *H. miracle-pistache.* The present study aims to investigate the phytochemical profile and total antioxidant capacity of *H. moserianum, H. miracle-pistache, H. perforatum, H. humifusum* and *H. maculatum*, to assess whether they are good sources of bioactives, with special interest in their hyperforin and hypericin content.

Polar solvents such as methanol and ethanol are extensively used to extract various compounds from plants [22]. The diversified polarities and chemical properties of bioactives influences their solubility in a solvent, which then affects the extraction yield. As solvent type affects antioxidant capacity and extraction yield, methanol 25% (v/v), ethanol 50% (v/v), and ethanol 70% (v/v) were used as solvents to maximize the yield of phytochemical compounds in the extracts.

RESULTS AND DISCUSSION

A thorough analysis of the phytochemical profile of five *Hypericum* species was assessed. In addition, the antioxidant activity of the ethanolic and methanolic extracts was performed by using the ABTS assay. The tested vegetal extracts were obtained by two distinct methods: ultrasound assisted extraction (UAE) and maceration (ME).

UAE is known to increase the yield of extracts with shorter extraction time. The acoustic cavitation destroys the cell walls and increases contact between the phytochemicals and the solvent, by reducing particle size. However, longer extraction periods with ultrasound (>20 minutes) can induce degradation of some phytochemicals such as polyphenols, resulting in a lower yield. Therefore, samples were subject to UAE treatment for 10 minutes only. Compared to UAE, extraction by maceration (ME) carries no phytochemical degradation risk and was applied to a separate batch of each sample, to ensure all phytochemicals were in conditions favourable for their extraction.

Evaluation of polyphenols

A complex phenolic profile was displayed by all five *Hypericum* species, as they contained most polyphenols including chlorogenic acid, hyperoside, isoquercitrin, quercitrin, quercetol, epicatechin, and rutoside. The results of the phytochemical analysis are summarized in Table 1 and are expressed as mg/100 g of dry weight (d.w.) plant material.

Concentrations of chlorogenic acid ranged from 2.214 mg/100 g d.w. in *H. perforatum* (sample 20) to 1350.072 mg/100 g d.w. in *H. miracle-pistache* (sample 27). Chlorogenic acid was the most abundant polyphenol in *H. humifusum*, *H. moserianum* and *H. miracle-pistache* (983.023 mg/100 g d.w., 1160.206 mg/100 g d.w. and 1350.072 mg/100 g d.w. respectively). *H. perforatum* had the least amount across all the extraction and solvent conditions.

Hyperoside was the most abundant polyphenol in *H. maculatum* and concentrations ranged from 33.782 mg/100 g d.w. (sample 12) to 1689.227 mg/100 g d.w. (*H. maculatum*, sample 8) across the five species. Methanolic extracts of each species yielded the highest hyperoside concentrations, regardless of the extraction method.

Rutoside was the most abundant polyphenol in *H. perforatum* (1143.468 mg/100 g d.w., sample 6) and was not detected in *H. miracle-pistache* under other conditions, except for sample 13 (1.005 mg/100 g d.w.). *H. humifusum* had the lowest concentration of rutoside (0.857 mg/100 g d.w., sample 7) and was below levels of detection in other conditions. Methanolic extracts of all five *Hypericum* species yielded the highest amount of rutoside, particularly in the UAE batch.

The concentration of isoquercitrin ranged from 7.593 mg/100 g d.w. in *H. perforatum* (sample 25) to 1130.729 mg/100 g d.w. in *H. maculatum* (sample 8). Epicatechin was present in all five *Hypericum* species, ranging from 14.517 mg/100 g d.w. in *H. humifusum*, (sample 16) to 290.829 mg/100 g d.w. in *H. maculatum* (sample 8). Quercitrin concentration ranged from 1.861 mg/100 g d.w. in *H. miracle-pistache* (sample 13) to 702.580 mg/100 g d.w. in

Table 1. Concentration of polyphenols (mg/100 g d.w.) in five Hypericum species extracts assessed with LC-MS analytical method

Sample Code	Vegetal species	Extraction method	Solvent (v/v)	Gentisic acid	Chlorogenic acid	p-Coumaric acid	Ferulic acid	Hyperoside	Isoquercitrin	Rutoside	Quercitrin	Quercetin	Luteolin	Kaempferol	Apigenin	Epicatechin	Catechin	Syringic acid	Gallic acid	Proto-catechuic acid	Vanillic acid
1	H. per	UAE	EtOH70%	x	4.026	3.691	2.225	204.717	72.627	578.471	84.496	222.270	0.951	5.391	ND	62.634	39.298	ND	1.209	7.372	3.491
2	H. hum	UAE	EtOH70%	x	587.061	ND	ND	114.844	54.904	x	37.757	18.690	2.058	x	ND	21.192	1.422	1.821	2.269	15.294	1.726
3	H. mac	UAE	EtOH70%	x	231.940	3.450	4.501	723.119	345.243	11.249	36.635	675.484	2.334	8.177	0.775	215.578	61.124	ND	1.933	18.467	3.754
4	H. m-p	UAE	EtOH70%	ND	1311.570	1.464	ND	488.019	352.948	ND	7.282	35.189	ND	0.947	ND	126.219	35.407	ND	ND	0.932	ND
5	H. mos	UAE	EtOH70%	ND	1059.876	ND	ND	89.344	50.127	220.858	495.244	8.542	ND	ND	ND	108.149	76.981	ND	0.442	1.284	ND
6	H. per	UAE	MtOH25%	x	4.328	4.895	3.590	526.373	218.721	1143.468	133.866	48.586	1.435	2.870	0.775	92.997	83.243	1.650	2.048	17.925	5.948
7	H. hum	UAE	MtOH25%	x	983.023	x	ND	308.791	155.999	0.857	93.844	5.624	3.648	x	ND	36.685	4.195	3.545	3.702	37.306	1.810
8	H. mac	UAE	MtOH25%	x	244.472	5.858	7.028	1689.227	1130.729	26.984	88.235	229.758	3.302	4.328	ND	290.829	95.160	1.333	2.803	31.918	6.401
9	H. mos	UAE	MtOH25%	ND	1160.206	x	ND	130.807	75.093	298.052	702.550	1.550	ND	ND	ND	158.731	107.665	1.765	ND	0.670	ND
10	H. per	UAE	EtOH50%	x	4.479	3.149	2.478	72.551	10.059	253.962	66.548	47.026	ND	x	ND	73.669	55.317	ND	2.247	10.614	4.831
11	H. hum	UAE	EtOH50%	x	669.952	x	x	83.436	32.867	x	37.383	52.367	1.712	1.411	ND	26.958	2.951	2.264	3.614	19.918	3.393
12	H. mac	UAE	EtOH50%	x	144.821	3.811	6.068	33.762	8.826	x	50.657	103.734	0.329	1.212	ND	196.712	56.025	ND	2.981	24.189	6.337
13	H. m-p	UAE	EtOH50%	ND	1302.511	1.284	x	380.213	252.162	1.005	1.861	83.529	ND	2.670	ND	117.755	38.118	ND	ND	1.614	ND
14	H. mos	UAE	EtOH50%	ND	1136.048	ND	ND	88.826	45.196	223.976	513.940	15.039	ND	0.253	ND	133.539	93.156	ND	ND	1.450	ND
15	H. per	ME	EtOH70%	x	3.346	3.571	2.630	202.436	70.161	588.863	100.949	302.762	1.228	6.356	0.873	46.722	39.601	ND	2.141	14.403	3.646
16	H. hum	ME	EtOH70%	x	687.165	ND	ND	131.222	62.764	x	45.796	31.445	2.472	0.850	ND	14.517	1.657	2.014	2.793	23.464	2.029
17	H. mac	ME	EtOH70%	x	184.449	4.293	5.815	485.842	219.337	9.616	46.731	794.800	2.403	9.958	ND	202.947	63.522	ND	3.305	24.730	5.927
18	H. m-p	ME	EtOH70%	ND	1254.799	1.404	x	448.110	322.743	ND	6.722	41.190	ND	1.212	ND	89.105	30.185	ND	0.519	1.440	ND
19	H. mos	ME	EtOH70%	ND	1126.385	ND	ND	94.837	52.265	236.000	526.092	13.002	ND	x	ND	93.292	72.588	ND	ND	1.415	ND
20	H. per	ME	MtOH25%	x	2.214	2.848	1.872	308.998	125.496	645.125	69.186	27.151	0.951	1.875	ND	47.044	32.647	0.614	1.338	9.726	3.508
21	H. hum	ME	MtOH25%	x	475.029	ND	ND	160.869	79.716	x	48.787	2.166	2.127	x	ND	19.005	2.682	1.987	1.844	18.492	1.204
22	H. mac	ME	MtOH25%	x	117.115	3.510	3.287	846.785	565.309	13.178	62.622	118.930	1.435	2.937	ND	155.528	48.945	0.924	1.091	19.044	3.760
23	H. m-p	ME	MtOH25%	ND	807.501	1.344	ND	476.720	335.380	ND	7.843	3.312	ND	ND	ND	108.943	34.474	2.068	ND	0.517	ND
24	H. mos	ME	MtOH25%	ND	943.314	ND	ND	103.960	60.295	238.572	561.614	2.101	ND	ND	ND	120.563	102.071	1.710	ND	0.697	ND
25	H. per	ME	EtOH50%	x	5.762	3.510	3.894	60.006	7.593	218.038	51.592	48.513	ND	x	ND	70.155	50.863	ND	2.494	11.784	4.824
26	H. hum	ME	EtOH50%	x	856.817	1.645	x	70.996	25.316	x	39.439	63.158	2.265	1.676	ND	28.001	3.199	2.530	4.339	22.533	5.298
27	H. m-p	ME	EtOH50%	ND	1350.072	1.404	6.877	341.755	215.839	x	5.226	125.261	ND	3.799	ND	98.820	33.295	ND	0.413	1.842	ND
28	H. mos	ME	EtOH50%	ND	1105.398	ND	ND	83.124	38.723	217.295	515.435	18.617	ND	0.452	ND	115.877	87.561	ND	ND	1.587	ND

H. per - H. perforatum, H. hum - H. humifusum, H. mac - H. maculatum, H. m-p - H. miracle-pistache and H. mos - H. moserianum. EtOH 70% - Ethanol 70% (v/v), EtOH 50% - Ethanol 50% (v/v), MtOH 25% - Methanol 25% (v/v).aftaric acid, caffeic acid, sinapic acid, myricetol and patuletin were not detected in any of the five species and therefore not represented on the results table. ND – Not determined, X – found in traces, identified on MS.

H. moserianum, (sample 9). With regards to the experiment conditions, UAE and methanolic extracts of all five *Hypericum* species had higher yields of isoquercitrin, quercitrin and epicatechin than their counterparts.

Regarding *H. humifusum*, Toiu *et al*. [23] reported lower chlorogenic acid concentration (<0.02 mg/100 g d.w.), but Nogueira *et al*. [24] reported a significantly higher amount (4.18 mg/100 g d.w.) than the present study. Toiu *et al*. [25] found higher levels of rutoside (1.4±0.09 mg/100 g d.w.) but reported similar levels of hyperoside (229.83±5.42 mg/100 g d.w.) and lower levels of quercitrin (27.08±2.64 mg/100 g d.w.) in ethanolic extracts.

Oniga et al. [25] reported significantly lower levels of chlorogenic acid (27.15±0.19 mg/100 g d.w.) in methanolic extracts of *H. maculatum*, similar levels of rutoside (11.00±0.1 mg/100 g d.w.) and hyperoside (545.14±2.96 mg/100 g d.w.). However, UAE extracted significantly higher hyperoside levels in the present study (sample 8).

The polyphenolic profile of *H. perforatum* reported by *Silva et al*. [26] was congruent the present study. However, higher levels of *p*-coumaric acid (32.2±0.16 mg/100 g d.w.) was reported by Wojdyło *et al*. [27] for *H. perforatum*. For *H. miracle-pistache*, Crockett *et al*. [28] only qualitatively reported the presence of isoquercitrin and quercitrin. Differences between the present study and comparative studies can be accounted for by factors such as genetic variation within the plant species, soil composition or geographical origins, which can affect phytochemical composition [29].

Evaluation of methoxyflavones

Three distinct methoxyflavones (hispidulin, hypericin, and hyperforin) were identified in the evaluated *Hypericum* species. The results are given in Table 2 and are expressed as mg/100 g of dry weight (d.w.) plant material.

The concentration of hispidulin ranged from 0.010 mg/100 g d.w. (*H. moserianum*, sample 28) to 0.432 mg/100 g d.w. (*H. maculatum*, sample 3). Hispidulin was absent in *H. moserianum* (except in sample 28) and *H. miracle-pistache*.

Hyperforin was absent in *H. humifusum* but was present in low concentrations in the other species, ranging from 4.583 mg/100 g d.w. in *H. miracle-pistache* (sample 4) to 0.022 mg/100 g d.w. in *H. moserianum* (sample 28). Similar results for ethanolic extracts *H. perforatum* were reported by Maggi *et al.* [30] Hyperforin was absent in all methanolic extracts, except *H. miracle-pistache* (sample 23) and *H. moserianum* (sample 9 and 24), suggesting methanol as the least favourable solvent for hyperforin extraction. However, other studies have successfully extracted hyperforin in methanol [32]. Ethanol 70% (v/v) yielded the highest amounts of hyperforin for both extraction techniques, presenting as the most favourable solvent.

Table 2. Concentration of hypericin and hyperforin (mg/100 g d.w.) in five Hypericum species

Sampl. code	*Vegetal species*	*Extraction method*	*Solvent (v/v)*	*Hispidulin*	*Hypericin*	*Hyperforin*
1	*H. per*	UAE	EtOH 70%	0.111	5.892	0.155
2	*H. hum*	UAE	EtOH 70%	0.061	0.884	ND
3	*H. mac*	UAE	EtOH 70%	0.432	5.695	0.051
4	*H. m-p*	UAE	EtOH 70%	ND	ND	4.583
5	*H. mos*	UAE	EtOH 70%	ND	ND	0.049
6	*H. per*	UAE	MeOH 25%	0.160	0.110	ND
7	*H. hum*	UAE	MeOH 25%	0.097	1.567	ND
8	*H. mac*	UAE	MeOH 25%	0.379	1.855	ND
9	*H. mos*	UAE	MeOH 25%	ND	ND	0.032
10	*H. per*	UAE	EtOH 50%	0.021	0.857	0.536
11	*H. hum*	UAE	EtOH 50%	0.053	0.181	ND
12	*H. mac*	UAE	EtOH 50%	0.063	2.829	0.082
13	*H. m-p*	UAE	EtOH 50%	ND	ND	0.091
14	*H. mos*	UAE	EtOH 50%	ND	ND	ND
15	*H. per*	ME	EtOH 70%	0.087	5.092	0.352
16	*H. hum*	ME	EtOH 70%	0.062	0.023	ND
17	*H. mac*	ME	EtOH 70%	0.255	6.419	0.134
18	*H. m-p*	ME	EtOH 70%	ND	ND	2.056
19	*H. mos*	ME	EtOH 70%	ND	ND	0.069
20	*H. per*	ME	MeOH 25%	0.064	ND	ND
21	*H. hum*	ME	MeOH 25%	0.056	1.631	ND
22	*H. mac*	ME	MeOH 25%	0.147	0.099	ND
23	*H. m-p*	ME	MeOH 25%	ND	ND	0.800
24	*H. mos*	ME	MeOH 25%	ND	ND	0.054
25	*H. per*	ME	EtOH 50%	0.019	1.482	0.357
26	*H. hum*	ME	EtOH 50%	0.064	0.260	ND
27	*H. m-p*	ME	EtOH 50%	ND	ND	0.062
28	*H. mos*	ME	EtOH 50%	0.010	ND	0.022

H. per - H. perforatum, H. hum - H. humifusum, H. mac - H. maculatum, H. m-p - H. miracle-pistache and H. mos - H. moserianum. ND – Not determined. UAE-ultrasound assisted extraction; ME-maceration. EtOH 70% - ethanol 70% (v/v), EtOH 50% - ethanol 50% (v/v), MtOH 25% - methanol 25% (v/v).

Hypericin was absent in *H. moserianum* and *H. miracle-pistache*. *H. maculatum* (6.419 mg/100 g d.w., sample 17) had the highest amount of hypericin, and *H. humifusum* (0.023 mg/100 g d.w., sample 16) had the lowest. Similar results for methanolic extracts of *H. maculatum* were reported by Mártonfi *et al*. [31] in the stamen (0.058 mg/g d.w.), but higher concentrations were found in the petals (0.096 mg/g d.w.). Ethanolic extracts (EtOH 70%) obtained by UAE of *H. perforatum* had a significantly higher amount of hypericin than *H. humifusum* (5.892 mg/100 g d.w., 0.884 mg/100 g d.w. respectively) in the present study. However, *Nogueira et al*. [26] reported the opposite and found higher concentrations of hypericin (0.24 mg/100 g d.w.) in *H. humifusum* than in *H. perforatum* (0.07 mg/100 g d.w.). Ethanol 70% (v/v) yielded the highest concentrations of hypericin (samples 1,3,15 and 17).

Considering the pharmacological and possible therapeutic use of hypericin and hyperforin, quantifying their concentrations in each *Hypericum* species were of special interest. Identifying more natural sources of these compounds could be used for more clinical studies. Interestingly, *H. miracle-pistache* had higher concentrations of hyperforin than *H. perforatum* (sample 4 and 18 compared to sample 1 and 15 respectively). This suggests that *H. miracle-pistache* could be more efficacious in treating mild to moderate depression with fewer chances of causing photosensitivity to the skin as no hypericin was detected in the species (samples 4 and 18). However, additional clinical research is required to verify this hypothesis.

Evaluation of sterols

The results for the identification and quantification of sterols in *Hypericum* species are summarized in Table 3. All sterols (except ergosterol) were identified in all five species in varying amounts. The concentration of stigmasterol ranged from 0.338 mg/100 g d.w. (sample 3) to 16.008 mg/100 g d.w. (sample 23). β-Sitosterol was the most abundant sterol found in all five *Hypericum* species, ranging from 0.582 mg/100 g d.w. (*H. moserianum*, sample 19) to 386.767 mg/100 g d.w. (*H. miracle-pistache*, sample 23). Campesterol concentrations ranged from 0.062 mg/100 g d.w. (*H. humifusum*, sample 7) to 1.778 mg/100 g d.w. (*H. maculatum*, sample 22).

Hernández *et al*. was the only previous study that identified campesterol, β-sitosterol and stigmasterol in *H. perforatum* and confirmed the absence of ergosterol [32]. However, the study did not quantify the sterols. This is the first report to identify and quantify sterols in the aerial parts of the assessed *Hypericum* species (except *H. perforatum*).

Table 3. The concentration of sterols (mg/100 g d.w.) in five Hypericum species extracts

Sample code	*Vegetal species*	*Extraction method*	*Solvent (v/v)*	*Stigmasterol*	*Beta-Sitosterol*	*Campesterol*
1	*H. per*	UAE	EtOH70%	2.658	75.335	0.582
2	*H. hum*	UAE	EtOH70%	1.349	13.345	0.202
3	*H. mac*	UAE	EtOH70%	0.338	9.306	0.068
4	*H. m-p*	UAE	EtOH70%	0.401	9.994	ND
5	*H. mos*	UAE	EtOH70%	ND	ND	ND
6	*H. per*	UAE	MtOH25%	0.755	13.309	0.140
7	*H. hum*	UAE	MtOH25%	0.378	2.956	0.062
8	*H. mac*	UAE	MtOH25%	ND	1.979	ND
9	*H. mos*	UAE	MtOH25%	15.808	365.674	0.767
10	*H. per*	UAE	EtOH50%	ND	ND	ND
11	*H. hum*	UAE	EtOH50%	ND	ND	ND
12	*H. mac*	UAE	EtOH50%	ND	1.637	ND
13	*H. m-p*	UAE	EtOH50%	ND	ND	ND
14	*H. mos*	UAE	EtOH50%	ND	ND	ND
15	*H. per*	ME	EtOH70%	0.872	14.695	0.160
16	*H. hum*	ME	EtOH70%	0.987	10.304	0.174
17	*H. mac*	ME	EtOH70%	0.619	16.592	0.112
18	*H. m-p*	ME	EtOH70%	6.811	170.129	0.625
19	*H. mos*	ME	EtOH70%	ND	0.582	ND
20	*H. per*	ME	MtOH25%	5.489	99.799	0.941
21	*H. hum*	ME	MtOH25%	3.338	35.187	0.547
22	*H. mac*	ME	MtOH25%	9.096	334.936	1.778
23	*H. m-p*	ME	MtOH25%	16.008	386.767	0.966
24	*H. mos*	ME	MtOH25%	9.643	214.720	0.507
25	*H. per*	ME	EtOH50%	ND	ND	ND
26	*H. hum*	ME	EtOH50%	ND	ND	ND
27	*H. m-p*	ME	EtOH50%	ND	ND	ND
28	*H. mos*	ME	EtOH50%	ND	ND	ND

H. per - H. perforatum, H. hum - H. humifusum, H. mac - H. maculatum, H. m-p - H. miracle-pistache and H. mos - H. moserianum. ND – Not determined. UAE-ultrasound assisted extraction; ME-maceration. EtOH 70% - ethanol 70% (v/v), EtOH 50% - ethanol 50% (v/v), MtOH 25% - methanol 25% (v/v).

A pattern was evident for all sterols with regards to extraction techniques and solvents used. Ethanol 50% (v/v) extracted no sterols in all five *Hypericum* species, except in *H. maculatum* where β-sitosterol (sample 12) was extracted. This suggested that ethanol 50% (v/v) was the least favourable solvent for sterol extraction. The richest extracts of each sterol were obtained using methanol 25% (v/v) with ME, followed by ethanol 70% (v/v) with UAE. For *H. moserianum*, methanol was the only solvent to successfully extract stigmasterol (sample 9 and 24, UAE and ME respectively). This suggested that the optimum conditions for β-sitosterol, campesterol and stigmasterol extraction was methanol and ME.

The evaluation of antioxidant activity

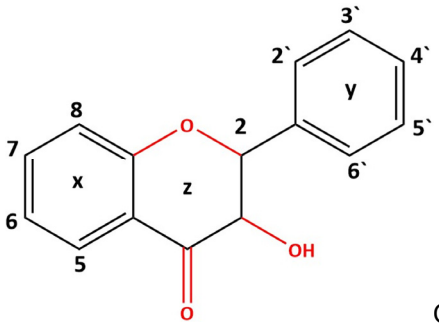
The ABTS assay is widely used to assess antioxidant capacity. Total antioxidant capacity (TAC) indicates the additive and synergistic action of all antioxidants present in a complex sample [33]. The results of this assay are expressed as Trolox equivalents (TE) per L of plant extract and are given in Table 4.

As the TAC for each *Hypericum* species were tested under a combination of six different conditions, the highest antioxidant level for each species were compared. The total antioxidant capacity ranged from 83.788 mM TE/L for *H. moserianum* (sample 14) and *H. maculatum* (sample 3 and 8) to 46.288 mM TE/L in *H. humifusum* (sample 7). Sample 9 for *H. moserianum* (15.227 mM TE/L) presents as an outlier as it was much lower than other results for *H. moserianum* in their respective conditions (samples 5,14, 19 and 28). Most of the extracts displayed the greatest antioxidant capacity in ethanol 50% (v/v). All *Hypericum* species (except *H. miracle-pistache*), displayed their highest antioxidant activity under UAE, however the ME counterparts also displayed similar antioxidant levels (see Table 4).

The strong TAC of *H. maculatum* (101.8 ± 1 μM TE/g) was supported by Zheleva-Dimitrova *et al*. [34] The same study reported significant scavenging ability for *H. perforatum* (81.2% ± 0.4 for ABTS), however the present study found moderate scavenging ability (58.788 mM TE/L, sample 10) for *H. perforatum*.

Flavonoids and polyphenols are major contributors to antioxidant activity, due to their ability to limit the oxidative degradation of lipids [35]. Phenolic compounds act as hydrogen donors, reducing agents and free radical scavengers [36]. The number of hydroxyl groups in the aromatic ring of a phenolic compound contributes to the difference in antioxidant activity between phenolic compounds. According to Zhang *et al.* [37], phenolic compounds with five hydroxyl groups (such as catechin and epicatechin),

present as the most active free radical scavengers. For flavonoids, the 4-carbonyl (ring Z), 3',4'-orthodihydroxy structure (ring Y), and 3,5-OH groups (creating a catechol-like structure in ring Z) ensures effective radical scavenging. The C2=C3 double bond in configuration with the 4-keto arrangement is responsible for electron delocalization from ring Y, resulting in increased radical-scavenging activity. The catechol structure in ring X compensates for antioxidant capacity in absence of the *o*-dihydroxy structure (ring Y) (see Figure 1) [27].



Quercetin (OH -3', 4', 5, 7)

Figure 1. General structure of flavonoids with annotations for quercetin. For quercetin, the catechol structure (ring Y), 2,3-double bond joined to the 4-carbonyl group (ring Z) allows delocalization of the phenoxyl radical electron to the flavonoid nucleus. Increase in resonance stabilization for electron delocalization is attributed to the 3-hydroxy group with the 2,3-doube bond, resulting in higher antioxidant value [27]

Glycosides formed from quercetin (a flavonoid) such as isoquercitrin, hyperoside and quercitrin, also display antioxidant activity. However, compared to quercetin, the glycosides are more water soluble due to the sugar portion of the molecules and a higher degree of absorption, leading to greater bioavailability in the body [38].

The highest concentration of quercitrin (794.800 mg/100 g d.w., sample 17) was found in *H. maculatum*, which also had a high concentration of hyperoside (1689.227 mg/100 g d.w., sample 8) and isoquercitrin (1130.729 mg/100 g d.w., sample 8) (Table 1). *H. moserianum* had high levels of chlorogenic acid (1160.206 mg/100 g d.w., sample 9) with high quercitrin levels (702.580 mg/100 g d.w., sample 9). The present study supports the correlation of antioxidant capacity to phenolic compounds as species that contained the highest concentrations of polyphenols and flavonoids corresponded with species that displayed high antioxidant activity.

Table 4. Trolox equivalent antioxidant capacity (TEAC) of five Hypericum species (expressed as mM TE/L - Trolox equivalent/L plant extract)

Sample code	***Vegetal species***	***Extraction method***	***Solvent (v/v)***	**TEAC *(mM TE/L)***
1	*H. per*	UAE	EtOH70%	35.682
2	*H. hum*	UAE	EtOH70%	44.015
3	*H. mac*	UAE	EtOH70%	83.788
4	*H. m-p*	UAE	EtOH70%	59.167
5	*H. mos*	UAE	EtOH70%	77.727
6	*H. per*	UAE	MtOH25%	56.894
7	*H. hum*	UAE	MtOH25%	46.288
8	*H. mac*	UAE	MtOH25%	83.788
9	*H. mos*	UAE	MtOH25%	15.227
10	*H. per*	UAE	EtOH50%	58.788
11	*H. hum*	UAE	EtOH50%	41.742
12	*H. mac*	UAE	EtOH50%	72.424
13	*H. m-p*	UAE	EtOH50%	70.909
14	*H. mos*	UAE	EtOH50%	83.788
15	*H. per*	ME	EtOH70%	47.424
16	*H. hum*	ME	EtOH70%	40.606
17	*H. mac*	ME	EtOH70%	74.318
18	*H. m-p*	ME	EtOH70%	60.303
19	*H. mos*	ME	EtOH70%	75.076
20	*H. per*	ME	MtOH25%	27.727
21	*H. hum*	ME	MtOH25%	36.818
22	*H. mac*	ME	MtOH25%	48.561
23	*H. m-p*	ME	MtOH25%	58.030
24	*H. mos*	ME	MtOH25%	78.485
25	*H. per*	ME	EtOH50%	53.106
26	*H. hum*	ME	EtOH50%	39.467
27	*H. m-p*	ME	EtOH50%	72.803
28	*H. mos*	ME	EtOH50%	78.485

H. per - H. perforatum, H. hum - H. humifusum, H. mac - H. maculatum, H. m-p - H. miracle-pistache and H. mos - H. moserianum. UAE-ultrasound assisted extraction; ME-maceration. EtOH 70% - ethanol 70% (v/v), EtOH 50% - ethanol 50% (v/v), MtOH 25% - methanol 25% (v/v).

CONCLUSIONS

In this study, *H. perforatum, H. humifusum, H. maculatum, H. miracle-pistache and H. moserianum* were investigated for their phytochemical profile and antioxidant activity. To the best of the authors knowledge, stigmasterol, campesterol, β-sitosterol and hispidulin were quantified for the first time in all assessed species. Among the polyphenols determined, chlorogenic acid, hyperoside, rutoside, isoquercitrin, quercitrin and quercetol were abundant across all five species. *H. maculatum* had the highest hypericin concentration and can be further investigated for photo-induced cytotoxic activity. *H. miracle-pistache* contained the highest hyperforin concentration which was of interest as *H. perforatum* is commercially marketed due to its antidepressant activity. However, with considerably higher amounts of hyperforin found in *H. miracle-pistache*, it may prove more effective than *H. perforatum* with even slimmer chances of photosensitivity as no hypericin was detected. However, more clinical research regarding its efficacy, therapeutic dose and safety for consumption is suggested before it can also be considered for commercial use. *H. maculatum* and *H. moserianum* exhibited the highest antioxidant activity, while *H. humifusum* presented with the lowest. Therefore, the *Hypericum* species serve as a useful source of bioactives, to the nutraceutical and pharmaceutical industry.

A key finding of this study was that *H. miracle-pistache* contained higher amounts of hyperforin than *H. perforatum* and hypericin was absent in the species. Several factors such as genetic variation within the plant species, soil composition or geographical origins can affect the phytochemical composition and consequently *in vitro* and *in vivo* activity. More specific to this study, the use of different extraction methods (UAE and ME) and different solvents influence the yield. Little comparative studies exist for *H. miracle-pistache*, so further phytochemical research should be conducted on the species to account for these factors and further certify the chemical composition of the species.

Further research into the clinical safety of ingesting *H. miracle-pistache* could also be conducted. This should highlight any adverse effects and a safe maximum amount which can be consumed daily. Finally, the clinical efficacy for mild to moderate depression could be assessed and compared to *H. perforatum*, with a safe and effective therapeutic dose identified. As *H. miracle-pistache* contained high amounts of chlorogenic acid and hyperoside, other clinical applications to conditions such as diabetes or Alzheimer's disease could be assessed.

EXPERIMENTAL SECTION

2.1 – Collection and identification of plant materials

Hypericum perforatum, Hypericum humifusum and *Hypericum maculatum* were gifted by Prof. Laurian Vlase. Fresh *Hypericum moserianum* and *Hypericum miracle-pistache* plants were store bought from a local supplier in Cluj-Napoca, Romania. Dr. Vlase Ana-Maria confirmed the authenticity of the plants, and a voucher specimen was deposited for each species at the Department of Pharmaceutical Botany, Faculty of Pharmacy. Aerial parts of each *Hypericum* species were prepared for lyophilisation by freezing for three hours at -20°C. The samples were lyophilised (SP Scientific Advantage 2.0, USA) at -55°C, 200 mTorr for one day and at -25°C, 200 mTorr for another four days. The samples were then milled into a powder and stored in amber glass bottles.

2.2 - Preparation of methanolic and ethanolic extracts

200 mg of each *Hypericum* species were mixed with the extraction solvents (2 ml of methanol 25% (v/v), ethanol 70% (v/v) and ethanol 50% (v/v)) in separate test-tubes for each solvent. An aliquot of each sample was filtered by centrifuge (Sigma 3-30 KHS, Sigma Laborzentrifugen GmbH, Germany) at the following settings: 25°C for 10 minutes at 10000 min^{-1}, 9168 g^{-1}, 12154 rotor. Ultrasound-assisted extraction (UAE) was performed (Transsonic 700, Elma D-788224, Singen, Germany) at 35 kHz, for 10 minutes every hour, for five hours daily, for nine days. For maceration (ME) the mixtures were shaken hourly, for five hours daily, for nine days.

2.3 - Determination of total antioxidant capacity

Total radical scavenging capacity was measured by ABTS assay previously described [39]. The Trolox equivalent antioxidant capacity (TEAC) assay reflects the ability of antioxidants to decolourise the blue-green 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonate)) ($ABTS^{\cdot+}$) radical, to a degree proportional to their concentration [40]. The ABTS reaction mixture consisted in 0.4 M acetate buffer (pH=5.8) and 10 mM $ABTS^{\cdot+}$ solution in 30 mM acetate buffer (pH=3.6). 12.5 µL of each extract was assessed spectrophotometrically (Analytik Jena Specord® 200 plus, Germany) at 660 nm, after 5 minutes reaction time with ABTS radical solution. Trolox, dissolved in a phosphate buffer (20 mmol/L, pH 7.4) with five-fold serial dilutions (0.05 mM – 1 mM), was used to make the calibration curve with a good regression coefficient (R^2>0.9972). The results of the assay were expressed in mM Trolox equivalents (TE)/L of plant extract.

2.4.1 – General HPLC and mass spectrometer equipment

The experiment was conducted on an Agilent 1100 HPLC series system (Agilent Technologies, Santa Clara, CA, USA) equipped with a G13311A gradient pump, column thermostat, G1322A binary degasser, G1313A auto sampler and a G1316AUV detector coupled with an Agilent 1100 mass spectrometer (MS) (LC/MSD Ion Trap VL).

2.4.2 - Qualitative and quantitative determinations of polyphenols

The HPLC-MS/MS method described by Vlase *et al.* [41,42] was adopted, with modifications of acetic acid replacing potassium phosphate in the mobile phase. 18 polyphenols were analysed: apigenin, caffeic acid, chlorogenic acid, caftaric acid, fisetin, ferulic acid, gentisic acid, hyperoside, isoquercitrin, kaempferol, luteolin, myricetol, patuletin, *p*-coumaric acid, quercitrin, quercetin, rutoside and sinapic acid. For chromatographic separation a reverse-phase analytical column was employed (Zorbax SB-C18, 100 x 3.0 mm i.d., 3.5 µm), with a mixture of methanol:acetic acid 0.1% (v/v) as the mobile phase and binary gradient [43]. The elution began with a linear gradient, starting with 5% methanol then 42% methanol, for 35 minutes. For the next 3 minutes isocratic elution followed with 42% methanol, rebalancing with 5% methanol in the next 7 minutes. The flow rate was 1 mL min^{-1}, the injection volume was 5 µL, with a column temperature of 48°C [44].

Compound detection was performed in UV and MS mode. Each compound was detected at wavelengths corresponding to the maximum absorption of their respective UV spectrum. Therefore, the UV detector was set at 330 nm for 17 minutes for polyphenolic acid detection, then 370 nm until 38 minutes to detect flavonoids and their aglycones. For quantitative determination, a calibration curve with a 0.5 µg-5 µg/ml range was made for each compound [45].

For six other polyphenols (catechin, epicatechin, vanillic acid, gallic acid, protocatechic acid and syringic acid) a Zorbax SB-C18 column, 100 x 3.0 mm i.d., 3.5 µm, with a mixture of methanol:acetic acid 0.1% (v/v) as the mobile phase and binary gradient was used to carry out chromatographic separation (starting with 3% methanol at 3 minutes, 8% methanol at 8.5 minutes, 20% methanol until 10 minutes then rebalance column with 3% methanol). The flow rate was 1 ml/min with a 5 µL injection volume [46].

The mass-spectrometer operated with an electrospray-ion source in negative mode (nebulizer 60 psi (nitrogen), drying gas nitrogen at 12 L/min flow rate, 360°C temperature, +3000 V capillary potential). The analysis mode was specific ion monitoring for polyphenolcarboxylic acids and AUTO MS for flavonoids and their aglycones. ChemStation and DataAnalysis software from Agilent USA processed the chromatographic data [47].

2.4.3 – Qualitative and quantitative determinations of methoxyflavones

The separation of methoxyflavones (acacetin, casticin, eupatilin, eupatorine, hispidulin and jaceosidin), was performed on a Zorbax SB-C18 analytic column (100 x 3.0mm i.d., 3.5 µm) at 48°C, in MS/MS, MRM mode [48]. Acetic acid 0.1% (v/v) and methanol made up the mobile phase beginning with 45% methanol and ending at 50% methanol for 8 minutes. The injection volume was 5 µL with a 0.9 ml/min flow rate and a gradient. The mass-spectrometer operated using an electrospray ion source in negative mode, under the following optimized conditions: nitrogen gas at 325°C, 12 L/min flow rate, 60 psi (nebulizer pressure), +2500 V capillary voltage and MS/MS specific ion monitoring analysis mode. To quantify each flavone in the extracts, the intensity of the selected ions from the mass spectra was considered. To construct the five point plot calibration curves, the standard solutions of each flavones were dissolved in methanol and successive dilutions were made in methanol:water (75:25 v/v).

2.4.4 - Qualitative and quantitative determinations of sterols

A previously described method was used to identify and quantify the phytosterols in *Hypericum sp.* extracts [49]. The following analytical standards were used: β-Sitosterol, campesterol, ergosterol and stigmasterol. Zorbax SB-C18 (100 x 3.0mm i.d, 3.5 µm) reverse-phase analytical column fitted with a Zorbax SB-C18 guard column was used for sterol separation, at 40°C under isocratic conditions in MS/MS, MRM mode. Acetonitrile:methanol (30:70 v/v) mixture made up the mobile phase, with a 1 ml/min flow rate and a 4 µL injection volume. The mass-spectrometer operated using an atmospheric pressure chemical ionization (APCI) interface, in positive mode, under these conditions: nebulizer 50 psi, gas (nitrogen) at 7 L/min flow rate, 250°C temperature, -4000 V capillary potential. Multiple analysis mode was used instead of single ion monitoring to limit background interference (MS/MS instead of just MS).

2.4.5 - Qualitative and quantitative determinations of hyperforin and hypericin

HPLC/ESI-MS (ion trap) analysis of hyperforin and hypericin was performed on a Zorbax SB-C18 (100 x 3.0 mm i.d, 3.5 µm) analytical column with a 0.2-micron filter (Agilent) at 45°C with a 1 ml/min flow rate [50,51].

Hypericin: The mobile phase was acetonitrile:ammonium acetate (1 mM) (50:50 v/v) solution with a 5 µL injection volume. The mass-spectrometer functioned using an electrospray ion source in negative mode, under the following optimized conditions: nitrogen gas at 325°C with a 12 L/min flow rate, nebulizer pressure of 60 psi and a 3000 V capillary voltage. The analysis mode was set to ion monitoring m/z 503 [52].

Hyperforin: For the mobile phase, 1 mM ammonium acetate solution was mixed with acetonitrile 35:65 (v/v). For detection, the mass-spectrometer operated using an electrospray ion source in negative MS/MS mode under the following optimized conditions: nitrogen gas at 350°C with a 12 L/min flow rate, nebulizer pressure of 60 psi and a 2500 V capillary voltage. The analysis mode used was transition monitoring m/z 535.4.

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