applied sciences

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Assessment and Comparison of Phytochemical Constituents and Biological Activities between Full Flowering and Late Flowering of *Hypericum perforatum* L.

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Abstract: This study assessed the impact of full and late flowering stages on the polyphenols and enzyme inhibitory properties of *Hypericum perforatum* from Poland. Recognizing the significance of phenolic compounds in disease prevention and melatonin's emerging protective role, we employed an UHPLC-MS/MS system to quantify 38 phenolic compounds, not typical of St. John's wort, and to develop a new method for melatonin quantification. Afterward, the extracts were tested for their antioxidant capabilities (using phosphomolybdenum, DPPH, ABTS, FRAP, CUPRAC and ferrous chelating assays). Moreover, we investigated enzymes (acetylcholinesterase, butyrylcholinesterase and tyrosinase) involved in neurodegenerative disorders and (α-amylase and α-glucosidase) in diabetes. This study recognized the importance of phenolic compounds in disease prevention and explored the emerging protective role of melatonin, taking into account the floral ontogeny of the plant. Indeed, the full-flowering plant contained the greatest concentration of phenolic compounds (a total of 65,276.5 μg/g): hyperoside (18,726.59 μg/g), isoquercitrin (11,895.02 μg/g) and delphindin-3.5-diglucoside (10,619.51 μg/g), and showed the highest inhibitory enzyme activity. Moreover, only full-flowering St. John's wort contained melatonin (40 ng/g). Our results offer additional perspectives on the chemical-biological characteristics of *H. perforatum* and scientific knowledge that testifies to the importance of considering plant growth conditions for the development of nutraceuticals.

Keywords: *Hypericum perforatum*; melatonin; UHPLC-MS/MS; antioxidant activity; polyphenols; biological activity; floral ontogeny

Citation: Piatti, D.; Marconi, R.; Caprioli, G.; Angeloni, S.; Ricciutelli, M.; Zengin, G.; Maggi, F.; Pagni, L.; Sagratini, G. Assessment and Comparison of Phytochemical Constituents and Biological Activities between Full Flowering and Late Flowering of *Hypericum perforatum* L. *Appl. Sci.* **2023**, *13*, 13304. https://doi.org/10.3390/app132413304

Academic Editor: Antony C. Calokerinos

Received: 19 November 2023
Revised: 5 December 2023
Accepted: 13 December 2023
Published: 16 December 2023

1. Introduction

In the treatment of different neurological diseases, such as Alzheimer's and Parkinson's, plant-based nutraceuticals represent a fundamental element today. The peculiarity of this study is the attention to the differences denoted in metabolic composition by the collection of the plant in different stages of floral ontogeny. The last few years have seen an expansion in market requests for natural bioactive compounds obtained from plants. Today, various herbal medicines obtained from medicinal plants through extraction or through other chemical and physical processes are used to improve human health. In particular, to verify the quality, efficacy and safety of each drug, authorization from the Italian Drugs Agency (AIFA) or the European Medicines Agency (EMA) is required. *Hypericum perforatum* L., today, represents a promising plant that has been studied and researched for its health-giving properties and, thanks to its history and therapeutic uses in the European

community over the last decade, it is a well-established herbal product in Italy. Nevertheless, it belongs to typical plants with restrictions, therefore, its daily consumption is carefully controlled to prevent unwanted pharmacological actions [1]. While some regions have declared *H. perforatum* to be a "noxious weed", it has been recognized as a beneficial herb since ancient times. Its use can be found in both historical and contemporary literature, as well as in official herbal compilations. It has been frequently reported in psychological disorder treatment, such as those of a psychiatric nature. Official herbal compendiums, from both ancient and modern times, mention the use of this substance to treat mental illness, thus attesting to its widespread use [2]. *H. perforatum* was recognized during the biblical era as "the rose of Sharon" and also St. John's wort, as it grew on the ground with the blood of the beheaded St. John the Baptist. Moreover, the plant blooms each June, on the days that approximately correspond to St. John's Day. In the Middle Ages, amulets were created with St. John's wort to be worn around the neck during battles. In this way, it was thought that soldiers were protected from harm. The plant was also used in everyday life as it was thought to cure depression and anxiety, thought to be caused by demons or witchcraft [3]. It is a perennial shrub (herb) belonging to the Hypericaceae; the genus includes more than 400 species. The plant grows 50 to 90 cm tall and can asexually and sexually reproduce. The distinctive morphological features of *H. perforatum* include the taproot system, woody stems, rhizomes, stolons, leaves with translucent glands throughout, and cymes of gold-yellow flowers that develop into dehiscent capsules for seed storage [4]. Despite the fact that *H. perforatum* is native to Europe, Western Asia and North Africa, it is now naturalized in North America, South America, Australia, India, New Zealand and South Africa through cultivation as a medicinal or garden plant. Today, St. John's Wort products are abundantly available in the current market, offered by various herb producers due to the plant's widespread cultivation worldwide. Consequently, the quality of *H. perforatum* preparations on the market can considerably vary, influenced by factors such as the use of different subspecies and varieties, the geographic locations of the raw material during collection, and variations in harvesting times corresponding to the different stages of plant development [5]. Considering the history of this plant, its secondary metabolites and pharmacological activities, *H. perforatum* could be an herbal dietary supplement for use worldwide. One of the uses of this plant is in its reduction of symptoms associated with moderate depression, such as anxiety, a reduction of appetite and energy, insomnia, hopelessness and suicidal ideation. Additionally, it has been seen to have anti-inflammatory and antiviral effects [6]. Also, other activities, such as antimicrobial, antioxidant, antitumoral and wound-healing properties, have been reported for this plant [7]. Furthermore, there is a strong correlation between depression and Alzheimer's or Parkinson's disease, since these conditions are commonly observed in patients with cognitive abnormalities and neurodegeneration [8]. Research has clearly demonstrated that the positive actions of *H. perforatum* depend on various bioactive constituents that act in a synergistic manner [9]. In fact, it has been reported that hyperforin and hypericin are the most active compounds of *Hypericum* extract for the treatment of depression but, due to the complexity of this disease, various bioactive compounds could be responsible for improvements in health [10,11]. Considering the hypothesis according to which the depression derived from a lack of dopamine, norepinephrine and serotonin, the three monoamine neurotransmitters in the brain, *Hypericum* extract seems to be active in this pathology because it inhibits the monoamine oxidases (MAOs) and, consequently, the oxidation of neurotransmitters in the brain. This could not be completely attributed to the actions of hyperforin and hypericin because they require low concentrations in the crude drug to obtain a similar effect and are present in the plant extract, together with many other molecules such as rutin, quercetin, chlorogenic acid and hyperoside [12–14]. In line with this, flavonoid fractions, also obtained from *H. perforatum* extract, have been reported to have antidepressant properties in experimental studies. Thanks to their action as antioxidant compounds, phenolic molecules have also been strongly correlated with oxidative damage and significantly contribute to the onset of neurodegenerative diseases

such as Parkinson's and Alzheimer's diseases [15]. In all of these diseases, *H. perforatum* seems to have a beneficial effect thanks to the inhibition of numerous enzymes, such as cholinesterases, tyrosinase, amylase and glucosidase, connected with the development of the illness. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two different enzymes involved in the degradation of acetylcholine and butyrylcholine, respectively. The lack of these enzymes leads to Alzheimer's disease. The defining feature of Parkinson's disease is a shortage of dopamine in the brain. Dopamine neurotoxicity, a process that contributes to the neurodegeneration commonly seen in Parkinson's disease, is connected with excessive activity of the tyrosinase enzyme. Moreover, the inhibition of α-amylase (α-AMYL) and α-glucosidase (α-GLUC) leads to control of postprandial plasma glucose elevation and hyperglycemia, obtaining a successful treatment strategy to manage diabetes [16]. In the development and progression of all the diseases mentioned above, numerous molecules such as naphthodianthrones, acyl-phloroglucinols, flavonoids and xanthones are involved [9]. Melatonin (N-acetyl-5-methoxytryptamine) is a powerful antioxidant molecule involved in the neurodegeneration process typical of Alzheimer's and Parkinson's, and is also used to treat insomnia typical in depressed patients [17]. Melatonin has been detected in numerous plant species, in particular, different authors have reported the presence of melatonin in *H. perforatum* [18–20]. Although, it is clear that the beneficial effects of *H. perforatum* derive from numerous molecules, there have been no studies available for this plant that have simultaneously investigated the presence of flavonoids, melatonin and the inhibitory power of various enzymes involved in numerous pathologies for the plant. The cooperative action of all compounds detected in the *H. perforatum* and the connection with beneficial effects were somewhat neglected. Furthermore, although it is known that numerous factors influence the quality of the plant and, consequently, its content of active metabolites, few studies have taken this into consideration when developing a nutraceutical.

Hence, the aim of this investigation was to compare the phytochemical profile and biological activities of *H. perforatum* L. collected in the same area of Poland in two stages of floral ontogeny, that is: "full" and "late" flowering phases. For this purpose, the polyphenols and the melatonin of *H. perforatum* extracts were quantified. A new method for the quantification of melatonin using UHPLC-MS/MS, and a new purification method for polyphenols, subsequently quantified with the UHPLC-MS/MS procedure reported in the literature, with some modifications, were used [21]. Moreover, the power of the inhibition of all the enzymes mentioned above (cholinesterases, tyrosinase, amylase and glucosidase), the radical scavenging activity, and the total amount of flavonoids and polyphenols were evaluated. As far as we know, considering all the literature, this study represents the first of its kind regarding the period of collection of the plant, the complete evaluation of the chemical composition of St. John's wort, the amount of melatonin and 38 polyphenols combined with the antioxidant activity and enzymatic inhibitory capacity. These study features allow us to understand the connection between the *H. perforatum* plant and its positive effect on various diseases, such as depression, but also Alzheimer's, Parkinson's and diabetes.

2. Materials and Methods

2.1. Sample Collection and Preparation

Three different samples of cultivated *H. perforatum* (flowers, including approx. 7 cm stems) from the same geographical areas of Poland were studied. Samples I and II were harvested at full bloom, with a time difference of roughly 1 week in between and compared with a sample harvested at late flowering (maturity). The collected plant materials were deprived of the damaged parts and subsequently dried in a mechanical, static drier, ventilated with air at 45 °C, for 24 h. Afterward, the plants were bagged and stored at room temperature in a dark room. The cultivation sites were in a flat area (80 m to 100 m asl) roughly 200 km north-west of Warsaw and the field had sandy soil.

2.2. Chemicals and Reagents

All the analytical standards used for this work were analytical or HPLC grade and mainly purchased from two companies: Merck (Milan, Italy) and PhytoLab (Vestenbergsgreuth, Germany). Separate individual solution was prepared for each substance (1000 mg L^{-1}). The pure standards were solubilized in LC-MS methanol and the ready solutions were stored at 4 °C. On the other hand, the anthocyanins were stored at a lower temperature, −15 °C. Day by day, additional solutions at different concentrations were created, starting from the standard working solutions. Dilutions were made using methanol. Formic acid of 99–100% was bought from J.T. Baker B.V. (Deventer, Holland). LC-MS methanol was supplied by Merck (Milan, Italy). Deionized water was further purified using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 µm syringeless filters, Phenomenex (Castel Maggiore, BO, Italy).

2.3. Preparation of Dried Extract

In accordance with Altun et al. [22], who demonstrated that methanol is the best solvent to acquire the highest content of phenols and enzymatic inhibitory activity from St. John's wort, the extraction was performed using the same extraction solvent. Each type of *H. perforatum* was pulverized and was put and extracted with 500 mL of MeOH, helped by ultrasounds (ultrasound-assisted extraction, UAE) for 20 min. The extract was totally dried under vacuum (60 mbar) at 30 °C by a rotavapor (Büchi R200, Labortechnik, Flawil, Switzerland) and then with an oil pump.

2.4. Spectrophotometric Assay

2.4.1. Sample Preparation

The dried extracts, prepared as previously described, were reconstituted in methanol (2 mg/mL) and subsequently employed for the following analysis.

2.4.2. Determination of the Total Phenolic and Flavonoid Content

The content of total phenolic in the extract was calculated using the Folin-Ciocalteu method reported by Fawzi Mahomoodally et al. [21]. For the tests, the data were given as gallic acid equivalents (mg GAEs/g dry extract) and rutin equivalents (mg REs/g dry extract).

2.4.3. Determination of Antioxidant Activity

Several chemical studies were conducted to evaluate the antioxidant characteristics of the extract. These tests involved several processes, such as radical scavenging, reducing power and metal chelating. For comparison, trolox and ethylenediaminetetraacetic acid (EDTA) were used as conventional antioxidant chemicals. The outcomes that were attained were represented as these chemicals' equivalents. Because CUPRAC (cupric-reducing antioxidant capacity) and FRAP (ferric-reducing ability of plasma) indicate the electron-donating capacity of antioxidant compounds, they were utilized in this investigation to comprehend reductive ability. Furthermore, the phosphomolybdenum method was realized to evaluate the total antioxidant capacity because of the reductive activity of both phenolic and non-phenolic compounds (e.g., ascorbic acid, tocopherol, etc.). Finally, the metal chelating activity, involved in lipid peroxidation and in other oxidation processes, was also performed. The assays were realized according to the method reported by Fawzi Mahomoodally et al. [21].

2.4.4. Enzyme Inhibitory Effects

For detecting the inhibitory effects on enzymes, including tyrosinase, α-glucosidase, α-amylase and cholinesterases, colorimetric enzyme inhibition assays were used. The assays were realized according to the method reported by Fawzi Mahomoodally et al. [21] with some modifications. The standard inhibitors, galantamine, kojic acid and acarbose, were used as controls. The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase

(BChE) was reported as mg galanthamine equivalents (GALAE)/g extract, while the inhibition of amylase and glucosidase was represented as acarbose equivalents (ACAE)/g extract. The expression for tyrosinase inhibition was as kojic acid equivalents (KAE)/g of dried extract.

2.5. Sample Purification

2.5.1. Polyphenols Purification

Polyphenol extract purification was carried out using a method similar to the one reported in the literature by Alonso García et al. [23]. Discovery DSC-18 solid-phase extraction tubes 500 mg, with a volume of 6 mL, in a pack of 30, from Merck (Milan, Italy), were activated with 5 mL of methanol and then conditioned with 10 mL of water without allowing the cartridge to dry out. Then, 50 mg of dry extract, as previously obtained, was solubilized in 10 mL of water, and then an aliquot of this solution (5 mL) was passed through the cartridge. Phenolic compounds were eluted with 10 mL of a solution of 80% methanol/water. The eluate was completely evaporated using nitrogen gas and then dissolved in 10 mL of methanol. The purified extract was injected into UHPLC-MS/MS after centrifugation and filtration with a 0.22 µm syringeless filter.

2.5.2. Melatonin Purification

A new procedure was developed for melatonin purification; 50 mg of each extract was dissolved in 10 mL of ultrapure water. The procedure was realized using Strata C18 cartridges (Strata C18-E, 500 mg/6 mL) (55 µm, 70 A), from Phenomenex, Bologna, Italy. Before purification, the cartridges were activated with 5 mL of methanol and conditioned with 5 mL of Milli-Q water. Then, 4 mL of the aqueous extract was loaded onto the cartridge, which was then washed with 2 mL of water and eluted with 12 mL of a solution of 80% methanol/water at a flow rate lower than 0.5 mL/min. The eluate was totally evaporated with nitrogen gas and the residue was dissolved in 4 mL of methanol. The purified extract was injected into UHPLC-MS/MS after centrifugation and filtration with a 0.22 µm syringeless filter.

2.6. Chromatographic System and Operating Conditions

2.6.1. UHPLC-MS/MS Triple Quadrupole Method for 38 Polyphenols

A method previously published by Mustafa et al. [24] was employed for UHPLC-MS/MS investigations, utilizing an Agilent 1290 Infinity series coupled with a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA). Both negative and positive ionization modes were used with the electrospray ionization (ESI) source. A Synergi Polar–RP C18 analytical column (250 × 4.6 mm, 4 µm) from Phenomenex (Chesire, UK) and a Polar RP security guard cartridge (4 × 3 mm ID) were used to separate the target compounds. The mobile phase was made up of (A) water and (B) methanol combined with 0.1% formic acid, flowing at a rate of 0.8 milliliters per minute in gradient elution mode. The composition of the mobile phase varied as follows: 0–1 min, isocratic condition, 20% B; 1–25 min, 20–85% B; 25–26 min, isocratic condition, 85% B; 26–32 min, 85–20% B. The injection volume was 2 µL. The column temperature was set at 30 °C, while the ionization source's drying gas temperature was 350 °C. The gas flow, nebulizer pressure and capillary voltage were 12 L/min, 55 psi and 4000 V, respectively. For detection, the dynamic-multiple reaction monitoring (dynamic-MRM) mode was employed, with the integration of dynamic-MRM peak areas for quantification. Quantitation utilized the most abundant product ion, while others served for qualification. Each compound had a specific time window (Δ retention time) set at 2 min. Details of the selected transitions for the analyzed compounds, including precursor ion, product ion, fragmentor, collision energy, retention time and delta retention time, are provided in Table 1.

Table 1. HPLC–MS/MS acquisition parameters (dynamic-MRM mode) used for the analysis of the 38 marker compounds.

No.	Compounds	Precursor Ion, *m*/*z*	Product Ion, *m*/*z*	Fragmentor, V	Collision Energy, V	Polarity	Retention Time (Rt, min)	Delta Retention Time (ΔRt)
1	Gallic acid	169	125.2 *	97	12	Negative	6.96	2
2	Neochlorogenic acid	353	191.2 *, 179	82	12, 12	Negative	9.52	2
3	Delphindin-3-galactoside	465.01	303 *	121	20	Positive	11.36	2
4	(+)-Catechin	289	245.2 *,109.2	131	8, 20	Negative	11.44	2
5	Procyanidin B2	576.99	576.99 *, 321.2	160	0, 32	Negative	12.41	2
6	Chlorogenic acid	353	191.2 *, 127.5	82	12, 20	Negative	12.42	2
7	p-Hydroxybenzoic acid	137	93.2 *	92	16	Negative	12.86	2
8	(-)-Epicatechin	289	245.1 *, 109.1	126	8, 20	Negative	13.03	2
9	Cyanidin-3-glucoside	449	287.3 *, 255.6	121	20, 20	Positive	13.14	2
10	Petunidin-3-glucoside	479.01	317 *, 302	121	20, 44	Positive	13.26	2
11	3-Hydroxybenzoic acid	137	93.2 *	88	8	Negative	13.59	2
12	Caffeic acid	179	135.2 *, 134.1	92	12, 24	Negative	13.65	2
13	Vanillic acid	167	152.4 *, 108.1	88	12, 20	Negative	14.32	2
14	Resveratrol	227	185 *	131	12	Negative	14.40	2
15	Pelargonidin-3-glucoside	433.01	271 *, 121	116	24, 50	Positive	14.52	2
16	Pelagonidin-3-rutinoside	579.01	271 *	145	32	Positive	14.56	2
17	Malvidin-3-galactoside	493.01	331 *, 315.1	121	20, 50	Positive	14.64	2
18	Syringic acid	196.9	182.2 *, 121.2	93	8, 12	Negative	15.28	2
19	Procyanidin A2	575	575 *, 285	170	0, 20	Negative	16.18	2
20	p-Coumaric acid	163	119.2 *, 93.2	83	12, 36	Negative	16.70	2
21	Ferulic acid	193	134.2 *, 131.6	83	12, 8	Negative	17.10	2
22	3,5-Dicaffeoylquinic acid	514.9	353.1 *, 191	117	8, 28	Negative	17.61	2
23	Rutin	609	300.2 *, 271.2	170	32, 50	Negative	17.73	2
24	Hyperoside	465.01	303 *, 61.1	97	8, 50	Positive	18.33	2
25	Isoquercitrin	463	271.2 *, 300.2	155	44, 24	Negative	18.36	2
26	Delphindin-3,5-diglucoside	462.9	300.1 *	165	24	Negative	18.38	2
27	Phloridzin	435.39	273 *, 167	155	8, 28	Negative	18.83	2
28	Quercitrin	446.99	300.2 *, 301.2	160	24, 16	Negative	19.61	2
29	Myricetin	316.99	179.1 *, 182	150	16, 24	Negative	19.61	2
30	Naringin	578.99	271.3 *, 151.3	170	32, 44	Negative	19.62	2
31	Kaempferol-3-glucoside	447	284.2 *, 255.2	170	24, 40	Negative	19.77	2
32	Hesperidin	611.01	303 *, 334.8	112	20, 12	Positive	20.19	2
33	Ellagic acid	301	301 *, 229	170	0, 24	Negative	21.41	2
34	trans-cinnamic acid	149	131.2	74	4	Positive	21.44	2
35	Quercetin	300.99	151.2 *, 179.2	145	16, 12	Negative	21.87	2
36	Phloretin	272.99	167 *, 123	116	8, 20	Negative	22.30	2
37	Kaempferol	287.01	153 *, 69.1	60	36, 50	Positive	23.84	2
38	Isorhamnetin	314.99	300.2 *, 196.1	145	16, 4	Negative	24.57	2

* These product ions were used for quantification.

2.6.2. UHPLC-MS/MS Triple Quadrupole Method for Melatonin

Using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA), equipped with an electrospray ionization (ESI) source operating in positive ionization mode, the method was conducted. The separation of target compounds occurred on a Sinergy Polar-RP 80 A (250 × 4.6 mm, 4 µm) from Phenomenex (Castel Maggiore, Bologna, Italy). A gradient elution with a flow rate of 0.8 mL/min was employed, utilizing a mobile phase consisting of a mixture of water (A) and methanol (B), both with 0.1% formic acid. The solvent composition was: 0–15 min, 40–85% B; 15–17 min, isocratic condition, 85% B; 17–22 min, 85–40% B. This was followed by a 10-min reconditioning of the column. A 2 µL injection volume was utilized, and the column temperature was maintained at 30 °C, while the ionization source's drying gas temperature was set at 350 °C. The gas flow, nebulizer pressure and capillary voltage were set at 12 L/min, 25 psi and 4000 V, respectively. Detection in "multiple reaction monitoring" (MRM) was performed, and quantification was based on the integration of MRM peak areas. For quantitation, the most abundant product ion, 174.2 m/z, was utilized, while 159.1 m/z was employed to confirm the analyte presence. The acquisition parameters for this analyte, including retention time, fragmentor and collision energy, are detailed in Table 2.

Table 2. HPLC-MS/MS acquisition parameters optimized for melatonin.

Compound	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Fragmentor (V)	Collision Energy (V)	Retention Time (Rt, min)	Polarity
Melatonin	233.11	174.2 *	78	12	11.16	positive
	233.11	159.1	78	28	11.16	positive

* The product ion was used for quantification, the other to confirm the analyte.

2.7. Statistical Analysis

XLSTAT (Version 16) was used for statistical analysis. All analyses were performed in triplicate ($n = 3$) and presented as mean ± standard deviation (mean ± SD). The significance level for the one-way analysis of variance (AVOVA) and Tukey's post hoc test was set at $p < 0.05$ when comparing sample differences.

3. Results and Discussion

3.1. Total Polyphenols, Flavonoids and Antioxidant Assays

The outcomes of the test conducted are listed in Tables 3 and 4. Considering that the methanol extract has been proven to be the most effective in obtaining an extract with good antioxidant activity, all extracts were prepared using this solvent. In both DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays, the strongest radical scavenging ability was found in full bloom (I-II) samples with results of 62.19 to 171.40 mg TE/g and from 160.97 to 363.58 mg TE/g, respectively. The higher radical scavenging activities on DPPH or ABTS assays of the investigated samples could be justified by the high concentrations of polyphenols in the same extracts. Similar to radical scavenging assays, using the CUPRAC and FRAP assays, the best activities were observed in the same extracts. Also, in this case, the obtained results from radical scavenging and reducing power assays could be explained by the higher concentration of phenolic compounds. The antioxidant activity of the extracts was in fact consistent with their content of flavonoids and phenolic compounds, ranging from 46.71 to 60.62 mg RE/g and from 37.91 to 68.16 mg GAE/g, respectively. Comparing the obtained results with those of other authors, the resulting TPC and TFC were similar to those reported by Sekeroglu et al. [25] and by Becker et al. [26], but lower than those reported by Kalogeropoulos et al. [27]. Nevertheless, the TPC and TFC values of *H. perforatum* L. are affected by a number of variables, including the extraction solvent, the altitude of selected habitats, etc. [28]. In addition, spectrophotometric methods are not sufficient to accurately quantify total polyphenols and flavonoids because they do not distinguish between target compounds and other possible interfering compounds occurring in a complex matrix such as a plant extract. Therefore, the same extracts were investigated in the contents of individual polyphenols using the HPLC-MS/MS system.

Table 3. Total phenolic and flavonoid content.

Samples	Total Phenolics Content (mg GAE/g)	Total Flavonoids Content (mg RT/g)
H. perforatum I	68.16 ± 1.85 ^{a}	51.82 ± 0.66 ^{b}
H. perforatum II	65.63 ± 2.19 ^{a}	60.62 ± 0.82 ^{a}
H. perforatum III	37.91 ± 0.13 ^{b}	46.71 ± 0.42 ^{c}

Values are reported as the mean ± S.D. of three parallel measurements. TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalent; RE: rutin equivalent. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

Table 4. Antioxidant properties of the tested extracts.

Samples	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Chel. ab. (mg EDTAE/g)	Pho. (mmol TE/g)
H. perforatum I	171.40 ± 2.73 [a]	363.58 ± 5.82 [a]	386.92 ± 12.97 [a]	207 ± 6 [a]	15.13 ± 0.4 [b]	2.35 ± 0.17 [a]
H. perforatum II	159.45 ± 0.44 [b]	310.33 ± 6.9 [b]	315.63 ± 14.8 [b]	194.98 ± 2.65 [b]	12.96 ± 0.35 [c]	2.49 ± 0.04 [a]
H. perforatum III	62.19 ± 0.32 [c]	160.97 ± 2.97 [c]	143.99 ± 1.64 [c]	75 ± 3.46 [c]	19.65 ± 0.5 [a]	1.86 ± 0.12 [b]

Values expressed are means ± S.D. of three parallel measurements. TE: Trolox equivalent; EDTAE: EDTA equivalent. Chel. ab., Chelating ability; Pho., Phosphomolybdenum. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

3.2. Enzyme Inhibitory Activities

In the present work, the inhibitory activities of extracts against cholinesterase (AChE and BChE), amylase, glucosidase and tyrosinase have been investigated. The results are summarized in Table 5. The theory of enzyme inhibition is a potent tool in drug development, enabling the precise regulation of enzyme activity to alleviate the pathological symptoms associated with widespread health issues. By comprehending the intricate interactions among various enzymes and their impacts on biological pathways, scientists can devise medications that specifically target certain areas and impede particular processes. This approach has already demonstrated considerable success in the treatment of conditions such as Alzheimer's disease, diabetes mellitus and obesity. There are numerous works in the literature that have reported that phenolic compounds have promising cholinesterase inhibitory activities [29,30]. Since *Hypericum* extract is rich in phenolic compounds, it can be considered a promising AChE and BChE inhibitor. The results of our *H. perforatum* samples showed a similar AChE and BChE inhibitory effect for all tested extracts, from 1.98 ± 0.01 to 2.36 ± 0.07 mg GALAE/g and from 1.35 ± 0.12 to 1.86 ± 0.18 mg GALAE/g, respectively. Samples exhibited moderate inhibitory activity for both α-glucosidase and α-amylase, according to the data of Dong et al. [31]. In particular, we detected smaller values in terms of α-amylase inhibition for extracts than α-glucosidase inhibition. The samples were also tested for their capacity to inhibit the tyrosinase enzyme. The extracts derived from the full bloom harvested plants have a greater tyrosinase inhibitory activity than that observed for the other extract, obtained from sample III (harvested at late flowering maturity). In particular, the extract of *H. perforatum* with higher tyrosinase inhibitory activity showed a value of 52.49 ± 2.31 mg KAE/g. Tusevski et al. [12] obtained similar results with the highest enzyme inhibitory properties in *H. perforatum* L., originating from the Republic of Macedonia. Overall, *H. perforatum* extracts displayed encouraging biological activities, which warrant further investigations aimed at developing biopharmaceuticals.

Table 5. Extracts tested with all enzyme inhibition activities.

Samples	AchE Inhibition (mg GALAE/g)	BchE Inhibition (mg GALAE/g)	Tyrosinase Inhibition (mg KAE/g)	Amylase Inhibition (mmol ACAE/g)	Glucosidase Inhibition (mmol ACAE/g)
H. perforatum I	2.36 ± 0.07 [a]	1.86 ± 0.18 [a]	51.39 ± 1.7 [a]	0.41 ± 0.006 [a]	3.54 ± 0.02 [b]
H. perforatum II	2.23 ± 0.03 [b]	1.35 ± 0.12 [c]	52.49 ± 2.31 [a]	0.37 ± 0.001 [b]	3.98 ± 0.07 [a]
H. perforatum III	1.98 ± 0.01 [c]	1.65 ± 0.14 [b]	9.56 ± 1.34 [b]	0.38 ± 0.007 [b]	3.88 ± 0.03 [a]

Values expressed are means ± S.D. of three parallel measurements. GALAE: Galatamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

3.3. UHPLC-MS/MS Analysis of Phenolic Compounds

A total of thirty-eight different polyphenolic compounds in three purified St. John's wort extracts were quantified (Table 6). Numerous papers have been written on *H. perforatum* extracts, however, most of them have only reported the analyses of some individual compounds, such as hyperforins and hypericins [32,33]. Among the total 38 polyphenols tested, 26 were detected and quantitated. The extracts were generally very rich in polyphenols and followed the results obtained with spectrophotometric assays; in fact, the extracts obtained from full bloom plants are richer in molecules than the sample III, with 65,275, 38,799 and 22,857 μg/g of dried weight extract, respectively. Considering the plant with the higher amount of polyphenols determined by UHPLC–MS/MS, among them, hyperoside, isoquercitrin and delphindin-3,5-diglucoside resulted as predominant, with their concentrations being 18,726.59, 11,895.02 and 10,619.51 μg/g of dried weight extract, respectively. It is clear that *H. perforatum* extract contains a good amount of isoquercitrin; Silva et al. [34] also studied the correlation between the phenolic content of a full ethanolic extract of *H. perforatum* and fractions with antioxidant potentials, and found the presence of this molecule. Our experimental work revealed that the *H. perforatum* II extract is predominantly constituted of rutin (9573.17 μg/g), quercetin (4195.62 μg/g), quercitrin (2069.36 μg/g), neochlorogenic acid (4095.9 μg/g) and procyanidin B2 (1184.41 μg/g). These findings are aligned with those reported by Jürgenliemk et al. [35], where hyperoside and rutin were two of the most important polyphenols in the extract and neochlorogenic acid occurred in smaller amounts. Rutin was found to be the most predominant compound in *H. perforatum* extract obtained from MAE extraction [36]. However, all of the researchers mentioned above focused their investigation on the major polyphenols occurring in this plant, and generally, different polyphenols compared with those analyzed in the present research. Moreover, Sarikurkcu et al. [37] also quantified 26 different compounds in *H. perforatum*, but only 12 analytes corresponded to those quantified in the present work. For those compounds, the reported values by Sarikurkcu et al. [37] were in agreement with the outcomes found in the current paper. Generally, quercetin is one of the main phenolic compounds in different species of Hypericum extract. Napoli et al. [38] analyzed 11 Hypericum species (*H. perforatum* L., *H. aegypticum* L., *H. androsaemum* L., *H. calycinum* L., *H. hircinum* L., *H. hirsutum* L., *H. montanum* L., *H. patulum* Thunb., *H. perfoliatum* L., *H. pubescens* Boiss., *H. tetrapterum* Fr.). In all species, except *H. androsaemum* and *H. hircinum*, where hydroxycinnamic acids were the most prevalent polyphenols, quercetin derivatives are one of the main phenolic compounds. However, the value we found for quercetin appears to be higher than all those reported in this article for all the species. Nevertheless, our results are consistent with data on *H. perforatum* obtained from Mandrone et al. [39]. Furthermore, our values of chlorogenic acid, hyperoside and quercetin were generally higher than the values found in *H. hircinum*, but lower than those found in *H. scruglii* by Mandrone et al. [39]. Multiple research studies have conducted a comparison of various polyphenols in different *Hypericum* species, thereby validating that a wide range of polyphenol concentrations could change in relation to numerous internal or external factors, including the phenological stage, the species of the plant, the genetic profile, the growing site, the harvest period and the type of soil in which the plant grows [40].

3.4. UHPLC-MS/MS Analytical Method Validation for Melatonin Quantification

Different parameters were evaluated for the analytical method validation, such as assessing recovery, linearity, the limit of detection (LOD), the limit of quantification (LOQ) and repeatability. Recovery of the purification step was evaluated by comparing the area of the standard before and after the purification process (Figure 1). Moreover, the recovery has been evaluated in the real matrix by spiking it with melatonin standard. In detail, the areas obtained after the purification of the fortified extract (spike), the purification of the normal extract of *H. perforatum* II (blank) and the standard solution at the same spiking concentration were compared. In particular, the percentage of analyte recovered (%R) was calculated by relating the difference between the area of the spike and the area of the blank

(St. John's Wort extract) to the area of the standard solution multiplied by 100, as shown in Formula (1). The purification gave excellent results, with a recovery of 91.7% and an RSD of 2.8%.

$$\%R = \frac{Area\ spike - Area\ blank}{Area\ standard} \times 100 \quad (1)$$

Table 6. Results of 38 polyphenols UHPLC-MS/MS triple quadrupole. Data were expressed in μg/g of the dried extract.

No.	Compounds	*H. perforatum* I	*H. perforatum* II	*H. perforatum* III
1	Gallic acid	93.28	140.36	50.80
2	Neochlorogenic acid	3892.00	4095.90	1680.42
3	Delphindin-3-galactoside	n.d.	n.d.	n.d.
4	(+)-Catechin	349.86	519.22	129.04
5	Procyanidin B2	1319.85	1184.41	260.01
6	Chlorogenic acid	282.52	612.38	203.74
7	p-Hydroxybenzoic acid	78.57	184.91	138.17
8	(-)-Epicatechin	519.49	551.41	117.56
9	Cyanidin-3-glucoside	n.d.	n.d.	n.d.
10	Petunidin-3-glucoside	n.d.	n.d.	n.d.
11	3-Hydroxybenzoic acid	n.d.	n.d.	n.d.
12	Caffeic acid	29.04	77.52	23.17
13	Vanillic acid	84.12	204.61	128.20
14	Resveratrol	n.d.	n.d.	n.d.
15	Pelargonidin-3-glucoside	n.d.	n.d.	n.d.
16	Pelagonidin-3-rutinoside	n.d.	n.d.	n.d.
17	Malvidin-3-galactoside	n.d.	n.d.	n.d.
18	Syringic acid	10.47	24.33	18.87
19	Procyanidin A2	84.68	83.99	64.29
20	p-Coumaric acid	38.56	108.93	44.55
21	Ferulic acid	6.16	13.46	5.57
22	3.5-Dicaffeoylquinic acid	n.d.	0.95	0.82
23	Rutin	5843.49	9573.17	2405.53
24	Hyperoside	10,743.95	18,726.59	6334.01
25	Isoquercitrin	6518.96	11,895.02	4028.77
26	Delphindin-3,5-diglucoside	5805.38	10,619.51	3616.57
27	Phloridzin	6.32	11.12	3.47
28	Quercitrin	851.13	2069.36	976.78
29	Myricetin	15.12	15.32	4.43
30	Naringin	n.d.	n.d.	n.d.
31	Kaempferol-3-glucoside	112.56	241.29	112.70
32	Hesperidin	32.58	n.d.	42.87
33	Ellagic acid	n.d.	n.d.	n.d.
34	trans-cinnamic acid	13.79	15.11	10.46
35	Quercetin	2007.43	4195.62	2433.79
36	Phloretin	0.49	0.43	0.15
37	Kaempferol	59.80	111.60	73.24
38	Isorhamnetin	n.d.	n.d.	n.d.
Total		38,799.59	65,276.51	22,857.19

n.d. not detectable. Relative standard deviation (RSD) for all compounds ranged from 2.33 to 8.27%.

The linearity was calculated by injecting different concentrations of the external standard of melatonin (0.0001–1 μg/mL). The calibration curves were constructed by plotting the analyte peak areas against the analyte concentrations and the respective determination coefficients (R^2) were measured. The observed linearity of the target compounds was evidenced by R^2 values equal to or more than 0.9921. The LOQ and LOD were calculated by injecting low concentrations of the standard and calculated the signal-to-noise ratio

(SNR) using MassHunter Qualitative Analysis B.04.00 software from Agilent Technology (Santa Clara, CA, USA). The concentration that generated a signal-to-noise ratio of 10 was assigned to LOQ, while those of 3 were assigned to LOD. The instrument showed excellent sensitivity since the LOD for melatonin was 0.00004 µg/mL and the LOQ was 0.0001 µg/mL. The repeatability was tested by injecting three standard mixtures (0.001, 0.01 and 0.1 µg/mL) three times on the same day and on two consecutive days. The intra-day and inter-day repeatability were calculated by measuring the relative standard deviation (RSD %) of the standard peak areas of the standard solutions injected on the same day or on different days, respectively.

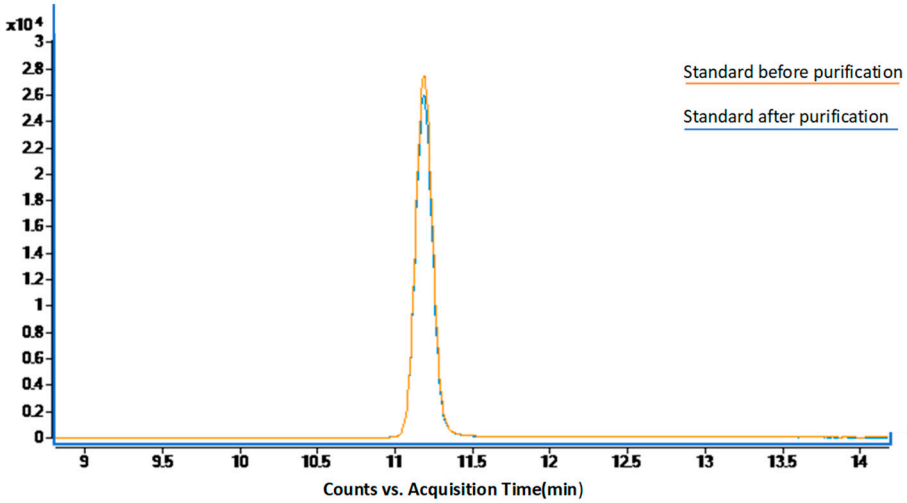

Figure 1. Overlay of UHPLC-MS/MS chromatograms of the 2 µg/mL melatonin standard solution before and after the purification process.

3.5. UHPLC-MS/MS Quantification of Melatonin

Although recent research has reported the production of neurohormone melatonin in St. John's wort plantlets [41], the levels of melatonin in all our studied samples were very low. In line with other authors [42], in only one of the three samples, the level of melatonin was quantifiable and corresponded to 40 ng/g. Nevertheless, the level of melatonin present in *H. perforatum* was not very high and it could be possible to enhance concentrations of the indoleamine melatonin by selecting the genetic variants of plants [20]. Furthermore, several variables have been seen to influence the melatonin level in the *Hypericum* plant, such as the plant species and the circadian clock [18]. The same study showed that good levels of melatonin were in fact found in *H. kouytchense* Lev. and *H. coris* L. compared to *H. perforatum*. They also confirmed that the melatonin concentration was not only affected by photoperiod, but also significantly by the wavelength and intensity of light applied. However, melatonin's regulation of genes and core pathways lacks precise information. This leaves vast potential for research across the plant kingdom to discover how melatonin affects essential life functions and find new approaches for industrial agriculture and plant cultivation [43].

4. Conclusions

The fight against numerous diseases, such as Alzheimer's and Parkinson's, finds fundamental support from the study of nutraceuticals. This starts from the knowledge of the plant and this article is solid proof of the importance of knowing the floral ontogeny of the plant for its impact on the phytochemical profile and biological activities of the extract.

Today, *H. perforatum* is a widely used herb around the world for its health benefits, especially in depression and mental disorders. The efficacy of its extract derives from the synergistic effect of the molecules that it contains. However, the beneficial actions of the molecules and all their synergistic effects are not all clear. In addition, further studies are necessary to clarify how the period of harvesting influences the content of

bioactive molecules and, consequently, the beneficial action of the plant extract. These results demonstrate that developing a nutraceutical it is essential to consider the floral ontogeny of the plant. Knowledge of floral ontogeny is fundamental, firstly because it can provide insights into the development of secondary metabolites in plants. These can in fact significantly vary, depending on the stage of maturation. Secondly, considering the period with the highest number of bioactive compounds could be useful to optimize its extraction methods. In our case, *H. perforatum* at the "full flowering" stage demonstrated great future potential to produce nutraceutical products for the treatment of health disorders. In fact, it contained a total of 65,276.51 µg/g polyphenols and 40 ng/g of melatonin. The present work revealed that all the tested extracts had interesting anti-cholinesterase and antioxidant properties, likely due to the rich content of polyphenols. To improve the knowledge of the impact of plant characteristics on the production of nutraceuticals, other variables should be taken into account, such as the growing soil and climatic conditions. In conclusion, these findings could be exploited for the further development of other nutraceuticals based on this plant, but this approach should be extended to other plants to consider to what extent plant characteristics can impact the final nutraceutical product.

Author Contributions: Conceptualization, D.P.; methodology, G.C. and G.Z.; software, M.R.; validation, D.P. and M.R.; formal analysis, D.P. and G.Z.; investigation, D.P. and R.M.; resources, L.P.; data curation, S.A.; writing—original draft preparation, D.P. and R.M; writing—review and editing, G.C.; visualization, L.P.; supervision, G.S. and F.M.; project administration, G.C. and F.M.; funding acquisition, L.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors thank the company FZL Sp. z.o.o., Kruszynek 10, 87-853 Kruszyn (Poland) for supplying the samples of *H. perforatum*.

Conflicts of Interest: Author Luca Pagni was employed by the company Indena SpA. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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