Rapid Communication

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Determination of the contents of bioactive compounds in St. John’s wort (Hypericum perforatum): Comparison of commercial and wild samples

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Abstract: St. John’s wort (Hypericum perforatum) is a medicinal plant with a rich history of traditional use. It has been shown to possess a range of beneficial health properties, including antioxidant and anti-inflammatory activities. In this study, the content of flavonoids and the antioxidant activity of commercially available dried and wild-grown samples were analyzed using the LC–MS/MS method. In addition, these samples were evaluated for their functional constituents, such as phenolic acids (ferulic, caffeic, chlorogenic, and gallic acids), quercetin, rutin, pseudohypericin, and hypericin using the liquid chromatography tandem mass spectrometry method. The most important antioxidant constituents in the samples analyzed were polyphenols with chlorogenic acid as the predominant compound. The content of the most important biocomponents with antidepressant activity was also analyzed. The results suggest that wild plants exposed to more stress factors have higher amount of compounds with antidepressant effects than plants grown in controlled conditions.

Keywords: Hypericum perforatum, hypericin, pseudohypericin, antioxidant properties, antidepression properties

1 Introduction

In the last decade, interest in a healthy lifestyle has increased significantly. Because of the ever-accelerating pace of life, many people are looking for sort of plant-inside chemicals that can raise their mood and help them with adapting stressful situations. It is well-known that in Chinese medicine many herbs were used to help people with a variety of illnesses and disorders that are an integral part of life [1]. St. John’s wort (Hypericum perforatum) is a medicinal plant with a rich history of traditional use that dates back to thousands of years. It is a perennial herb that belongs to the Hypericaceae family [2]. It grows up to 1 m in height and has small yellow flowers with five petals. The plant is known for its ability to grow in a wide range of conditions, from dry pastures to wetlands, and it is commonly found in fields and along roadsides. This herb is native to Europe, but is now found in many regions around the world [3].

St. John’s wort contains a diverse range of bioactive compounds, including hypericin, hyperforin, and various flavonoids such as quercetin, rutin, and kaempferol [4]. Hypericin and hyperforin are believed to be the main constituents responsible for the antidepressant activity. Hypericin is a photosensitive compound that is believed to inhibit the reuptake of certain neurotransmitters, such as serotonin, dopamine, and noradrenaline, in the brain. Hyperforin, on the other hand, has been shown to increase the levels of these neurotransmitters in the brain, leading to an improvement in mood [5].
Flavonoids are a second group of naturally occurring bioactive compounds found in St. John’s wort [6–8]. Flavonoids have been shown to possess a range of beneficial health properties, including antioxidant and anti-inflammatory activities. The antioxidant activity of flavonoids is due to their ability to scavenge free radicals, which are unstable molecules that can cause oxidative damage to cells and tissues. Free radicals are produced naturally by the body as a result of metabolism, but can also be generated by external factors such as pollution, radiation, and cigarette smoke [9]. When free radicals accumulate in the body, they can cause damage to DNA, proteins, and other cellular components, leading to various health problems such as cancer, heart disease, and ageing [10]. Flavonoids act as antioxidants by neutralizing free radicals and protecting cells and tissues from oxidative damage. They do this by donating an electron to the free radical, which stabilizes it and prevents it from causing further damage. In addition to their antioxidant properties, flavonoids also possess anti-inflammatory activity [11,12]. Inflammation is a natural response of the immune system to injury or infection, but when it becomes chronic, it can cause a range of health problems such as arthritis, diabetes, and heart disease [13,14]. Flavonoids have been shown to inhibit the production of inflammatory molecules such as cytokines and prostaglandins, which are responsible for the inflammatory response. Additionally, flavonoids can inhibit the activity of enzymes such as cyclooxygenase and lipoxygenase, which are involved in the production of inflammatory molecules. By inhibiting these enzymes, flavonoids can reduce inflammation and alleviate associated symptoms, such as pain and swelling [15].

Taking into account the aforementioned, in this study, the content of flavonoids and the antioxidant activity of commercially available wild St. John’s wort plant were analyzed. Furthermore, these samples were evaluated for their functional constituents, such as phenolic acids (ferulic, caffeic, chlorogenic, and gallic), quercetin, rutin, pseudohypericin, and hypericin, using the LC–MS/MS method. The antioxidant activity of the plants was also evaluated.

2 Materials and methods

2.1 Plant materials and extraction procedure

The first sample (denoted as Hp1) was a wild-type plant in the Wielkopolska region in Poland (52.37466002113175, 17.03565025813746). The next samples (Hp2–Hp6) were obtained from five commercial suppliers and were available in the Polish market.

All samples were ground to powder in a laboratory mill. An amount of 250 mg of samples was then extracted with 5 mL of 99% methanol for 30 min with centrifugation at 320 rpm (Cimarec i Poly 15 Multipoint Stirrer, Thermo Fisher Scientific, USA) for the whole extraction time. The supernatants were decanted and filtered (0.22 μm). For every plant three methanol extracts were prepared, giving 18 extracts in total (Figures 1 and 2). The extracts prepared in this way were immediately used for analysis.

2.2 Total flavonoid content

The total content of flavonoids was determined by the method described by Dalli et al. [16]. A 1 mL of distilled water and 50 μL of NaNO3 (5%, w/v) were added to 200 μL of the extract and waited for 5 min. Then 120 μL of AlCl3
(10%, w/v) was added and a further 6 min was allowed. After this, 400 μL of NaOH (1 M) was added. Finally, water was added to the samples so that their final volume was 3 mL. The amount of flavonoids was determined by the standard curve technique, which was prepared for the quercetin solution. A standard was prepared in the concentration range of 4–73 µg/mL. Prior to analysis, the standard solutions were subjected to the same procedure as the extracts. The results were presented as mg per g of dry plants [17].

2.3 Antioxidant activity

The antioxidant activity was assessed with the ABTS radical (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) [18,19]. To generate the radical cation, potassium persulfate (66.3 mg) was mixed with a solution of ABTS (360.3 mg) in 100 mL of distilled water and left for 48 h in the dark. The standard solution from which the curve was made was Trolox (16.3 mg/5 mL of methanol) prepared in the concentration range of 0.005–0.12 µmol/5 mL. The absorbance corresponding to the Trolox content was calculated from the formula:

\[ A = A_1 - A_0, \]

where \( A_1 \) is the absorbance of sample and \( A_0 \) is the absorbance of control.

From the methanolic extracts, 100 µL were taken into 5 mL flasks, 150 µL of ABTS** were introduced, and then supplemented with distilled water. After 6 min of storage in the dark, the absorbance of solutions was measured at 725 nm. On the basis of the equation of the obtained standard curve, the concentration of antioxidants in the extracts was calculated and expressed in µg of Trolox per 5 mL, which was converted to the weight of dried linden.

2.4 Determination of phenolic acids by LC–MS/MS

The determination of phenolic acids (ferulic, caffeic, chlorogenic, and gallic), as well as quercetin, rutin, pseudohypericin, and hypericin was performed with an UltiMate 3000 RSLC (Dionex, Thermo, USA) coupled with an API 4000 QTRAP triple quadrupole mass spectrometer with electrospray ionization (from AB Sciex, Foster City, CA, USA) in negative ionization modes (LC–MS/MS). A Luna C18 analytical column [20,21] (150 mm × 2.0 mm, particle size 3 µm particle size) from Phenomenex, USA was used for the chromatographic separation of these compounds [16,22]. The column temperature was maintained at 35°C and the injection volume was 5.0 µL. For the LC–MS/MS analysis of caffeic, ferulic, chlorogenic, and gallic acids, the mobile phase was prepared from Milli-Q water containing 5 mmol/L ammonium acetate (component A) and methanol (component B). The gradient program was 50% B from 0 to 2.5 min, increased to 100% B in 3 min and lasted 0.5 min; the flow rate was 0.20 mL/min. For the analysis of quercetin, rutin, pseudohypericin, and hypericin, the mobile phase was a gradient prepared from water with 0.8% CH₃COOH and 5 mM CH₃COONH₄ (component A) and acetonitrile (component B). The gradient program was 20% B from 0 min, increased to 100% B in 25 min and lasted 10 min; the flow rate was 0.2 mL/min. A post-run time was set at 4.0 min for column equilibration before the next injection. The operating conditions for mass spectrometry for all acids were as follows: curtain gas 10 psi, nebulizer gas and auxiliary gas 40 psi, source temperature 400°C (500°C for quercetin, rutin, pseudohypericin, and hypericin), ion spray voltage –4,500 V, and collision gas set to medium. Quantitative analysis of the compounds was performed in multiple reaction monitoring (MRM) mode. For analytes, one transitions of the deprotonated molecular ion and their respective ion product. These transitions (m/z) with associated declustering potentials (DP), collision energies (CE), and collision cell exit potential (CXP) are shown in Table 1. The quantification of phenolic acids was made by comparing the peak areas with those of the standards. The phenolic acid content in methanol extracts was determined by the multiple standard addition technique. For this purpose, four solutions of the sample
were prepared for each of the extracts, one without the addition of standard and three with the addition of standard at three concentration levels. For methanolic extracts, the concentrations of the standard solutions of phenolic acids added (ferulic, caffeic, and gallic) were, respectively, 0.005, 0.01, and 0.025 µg/mL. In the case of chlorogenic acid, standard additions of the mixture of chlorogenic acid were used with the following concentrations: 0.025, 0.05, and 0.1 µg/mL. The content of quercetin, rutin, pseudohypericin, and hypericin in methanol extracts was calculated using a standard calibration curve [23,24].

2.5 Validation of the LC–MS/MS methods

For the LC–MS/MS method, validation parameters were determined, i.e., limit of detection (LOD), limit of quantification (LOQ), and the linearity range. The LOD for all analytes was defined as the concentration that yielded S/N (signal/noise) ratio greater than or equal to 5, and the LOQ defined as the concentration that yielded S/N ratio greater than or equal to 10. Linearity was determined for a series of standard solutions of all analyzed compounds’ content in the concentration range from 0.000025 to 1 µg/mL.

2.6 Statistical analysis

Statistical analysis of the data was performed with Statistica 13 (Dell Software Inc., USA) software. All measurements were studied using one-way analysis of variance independently for each dependent variable. A post hoc Tukey honest significant difference multiple comparison tests were used to identify statistically homogeneous subsets at α = 0.05.

3 Results

The total flavonoids in the analyzed extracts showed that the highest content was found in Hp1, sample taken from the wild environment (Table 2). Most commercial samples contained similar amounts of flavonoids, ranging from 13.04 to 9.52 mg/g, but the content in Hp2 was much lower and amounted to only 5.38 mg/g of dry mass. Surprisingly, however, it turned out that the highest antioxidant activity was shown by the Hp2 extract (53.74 ± 1.49 mg/g), which contained the least flavonoids. The richest in antioxidants was the extract made from the Hp3 sample (53.74 ± 1.49 mg/g). The lowest values for this detection for extracts were designated for an extract made from sample Hp5 (14.70 ± 2.03 mg/g). The complete result of this research is presented in Table 2.

For the applied methods for the determination of phenolic acids and quercetin, rutin, pseudohypericin, and hypericin, the LOD, LOQ, and linearity range were determined based on the data contained in Section 2.4 The equation of the calibration curves, as well as the LOD and the LOQ are shown in Table 3. Good linearity was achieved with correlation coefficients of no less than 0.9794.

To determine the amounts of quercetin, rutin, hypericin, and pseudohypericin, LC–MS/MS methods have been used. The highest amounts of hypericin and pseudohypericin had been found in a sample collected from a natural environment, Hp1. Quercetin and rutin have been found in every sample, but in very low concentration in comparison with pseudohypericin. The highest concentrations of rutin have been found in Hp4 and Hp1. For quercetin, the highest

Table 2: Flavonoid content and antioxidant activity of analyzed samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flavonoid content (mg/g)</th>
<th>Antioxidant activity (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp1</td>
<td>19.96 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.69 ± 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp2</td>
<td>5.38 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.74 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp3</td>
<td>10.20 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.85 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp4</td>
<td>9.52 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35.12 ± 1.74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp5</td>
<td>9.14 ± 0.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.70 ± 2.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hp6</td>
<td>13.04 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.52 ± 1.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values marked with the same letter do not differ significantly p > 0.05.
concentrations were found in the Hp5 samples. The results are presented in Table 4.

The content of four phenolic acids found in St. John’s wort was also determined, and the results are presented in Table 5. In each of the extracts analyzed, the dominant acid was chlorogenic acid, and its content was characterized by high variability, from 99.20 µg/g for Hp2 to 3,550 µg/g for Hp5. The largest total quantity of phenolic acids was found in Hp5 and the lowest in Hp2.

### 4 Discussion

As mentioned above, *H. perforatum* L. is a source of many bioactive compounds, making it an interesting raw material widely used in the food industry [25]. The development and health of crops can be affected by different stress factors. These factors can be abiotic, such as temperature and water availability, or biotic, such as pests and diseases [26]. Plants have a natural defense against biotic stress through secondary metabolites that help protect them from damage and negative effects on their growth. Plants can produce secondary metabolites, which are compounds that are not essential for their basic metabolism but can help protect them from harm. These include alkaloids, terpenoids, and phenolic compounds. Flavonoids and, in general, polyphenolic compounds are such metabolites that are responsible for protecting plants against adverse external conditions and pathogenic infections [27]; therefore, the highest content was observed in the Hp1 sample. Other plants, grown under controlled conditions, are not exposed to numerous stress factors, so the synthesis of compounds responsible for protection was at a lower level [28]. Cultivation in controlled conditions, providing protection against various pests, but also ensuring optimal watering, means that plants synthesize the aforementioned secondary metabolites to a lesser extent, and their content is varietal-dependent [29]. However, no relationship was found between the total content of flavonoids and the antioxidant activity. Despite the lowest flavonoid content, Hp2 was characterized by the highest antioxidant activity. The use of the popular ABTS reagent allows the study of total antioxidant activity [19]. The radicals generated during the reaction are blue-green in color and show maximum absorbance at the following wavelengths: 417, 645, 734, and 815 nm. The antioxidants present in the test sample reduce the cation radical depending on the reaction time, as well as their activity and concentration, as a consequence, a decrease in color intensity can be observed in proportion to the content of antioxidants. The advantage of this method is that it allows the determination of the antioxidant capacity of both hydrophilic and

### Table 3: Linearity range, detection, and quantification of selected phenolic acids and salvianolic acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity range (µg/mL)</th>
<th>Curve equation</th>
<th>Correlation coefficient ((R^2))</th>
<th>LOD (µg/mL)</th>
<th>LOQ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>0.00025–1</td>
<td>(y = 4 \times 10^6 x + 40,149)</td>
<td>0.997</td>
<td>0.0001</td>
<td>0.00025</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.0005–1</td>
<td>(y = 2 \times 10^6 x + 11,213)</td>
<td>0.996</td>
<td>0.0001</td>
<td>0.0005</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.001–0.5</td>
<td>(y = 3 \times 10^6 x + 41,456)</td>
<td>0.988</td>
<td>0.0005</td>
<td>0.001</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.0025–0.5</td>
<td>(y = 7 \times 10^5 x + 15,626)</td>
<td>0.983</td>
<td>0.001</td>
<td>0.0005</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.025–1</td>
<td>(y = 2 \times 10^5 x - 74,716)</td>
<td>0.994</td>
<td>0.0025</td>
<td>0.025</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.0025–1</td>
<td>(y = 4 \times 10^4 x - 30,174)</td>
<td>0.9995</td>
<td>0.0005</td>
<td>0.0025</td>
</tr>
<tr>
<td>Pseudohypericin</td>
<td>0.01–0.5</td>
<td>(y = 282,675x - 30,595)</td>
<td>0.9794</td>
<td>0.001</td>
<td>0.01</td>
</tr>
<tr>
<td>Hypericin</td>
<td>0.01–0.25</td>
<td>(y = 5 \times 10^3 x - 442,488)</td>
<td>0.9955</td>
<td>0.0005</td>
<td>0.01</td>
</tr>
</tbody>
</table>

LOD, limit of detection; LOQ, limit of quantification.

### Table 4: Contents of quercetin, rutin, hypericin, and pseudohypericin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quercetin (mg/g)</th>
<th>Rutin (mg/g)</th>
<th>Hypericin (mg/g)</th>
<th>Pseudohypericin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp1</td>
<td>1.51 ± 0.01d</td>
<td>12.10 ± 1.12b</td>
<td>1.59 ± 0.04a</td>
<td>201.84 ± 4.44a</td>
</tr>
<tr>
<td>Hp2</td>
<td>0.35 ± 0.06a</td>
<td>0.98 ± 0.10f</td>
<td>0.25 ± 0.01df</td>
<td>17.30 ± 1.15a</td>
</tr>
<tr>
<td>Hp3</td>
<td>0.62 ± 0.01d</td>
<td>6.81 ± 0.71d</td>
<td>0.28 ± 0.05df</td>
<td>49.91 ± 3.68d</td>
</tr>
<tr>
<td>Hp4</td>
<td>1.12 ± 0.08a</td>
<td>15.34 ± 0.09a</td>
<td>0.49 ± 0.08a</td>
<td>71.68 ± 1.27b</td>
</tr>
<tr>
<td>Hp5</td>
<td>2.10 ± 0.28a</td>
<td>8.53 ± 0.11c</td>
<td>0.78 ± 0.03b</td>
<td>64.81 ± 3.18c</td>
</tr>
<tr>
<td>Hp6</td>
<td>1.07 ± 0.01c</td>
<td>4.74 ± 0.05b</td>
<td>0.21 ± 0.03b</td>
<td>43.20 ± 0.70d</td>
</tr>
</tbody>
</table>

Values marked with the same letter do not differ significantly \(p > 0.05\).
The second most abundant phenolic acid in St. John’s wort is caffeic acid. Like other polyphenols, it is believed to have many health benefits related to its antioxidant properties, including the prevention of inflammation, cancer, neurodegenerative diseases, and diabetes [40]. Nevertheless, according to published data, the therapeutic dose for caffeic acid is from 1 mg per day, and the recommended intake doses vary significantly between different diseases [41,42]. In the case of the analyzed samples of St. John’s wort, the highest content was found in Hp5, but even in this case, one infusion will provide only about 20% of the above-mentioned requirement.

St. John’s wort in natural medicine is associated with neurological activity. The most important compound in this work was pseudohypericin and hypericin, which have antidepressant properties. The content of these two chemicals depends not only on the variety of St. John’s wort, but also to a large extent on the environmental conditions in which St. John’s wort was grown [43]. Wild St. John’s wort has been found to be the best source of pseudohypericin and hypericin. Like flavonoids, both compounds are secondary metabolites, and their biosynthesis takes place on the polyketide pathway. In addition to oxidation, light also plays an important role in this reaction [44,45]. St. John’s wort that grows in the wild is exposed to a greater number of stress factors, including abiotic stress related to light and drought, which probably increased the content of pseudohypericin and hypericin. Consumption of 0.4–2.5 mg of hypericin has an antidepressant effect documented in clinical trials [46], so the consumption of one infusion of each analyzed herb can provide a therapeutic dose of this compound. It is worth noting, however, that the highest content was recorded for wild-growing St. John’s wort, so it seems reasonable to obtain this raw material from natural habitats.

5 Conclusions

St. John’s wort (H. perforatum) is a medicinal plant with a rich history of traditional use and contains a diverse range of bioactive compounds, including hypericin, hyperforin, and various flavonoids such as quercetin, rutin, and kaempferol. The study found that wild plants exposed to more stress factors have higher amounts of compounds with

Table 5: Phenolic acid content

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gallic acid (µg/g)</th>
<th>Caffeic acid (µg/g)</th>
<th>Ferulic acid (µg/g)</th>
<th>Chlorogenic acid (µg/g)</th>
<th>Sum of phenolic acids (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp1</td>
<td>5.79 ± 2.28d</td>
<td>44.90 ± 9.67c</td>
<td>23.26 ± 2.43b</td>
<td>2549.70 ± 89.38b</td>
<td>2623.65</td>
</tr>
<tr>
<td>Hp2</td>
<td>35.56 ± 7.75b</td>
<td>22.65 ± 12.22d</td>
<td>29.34 ± 2.87b</td>
<td>99.20 ± 5.52a</td>
<td>186.75</td>
</tr>
<tr>
<td>Hp3</td>
<td>35.47 ± 2.21b</td>
<td>72.77 ± 19.04ab</td>
<td>41.30 ± 15.86a</td>
<td>1992.05 ± 7.94c</td>
<td>2141.59</td>
</tr>
<tr>
<td>Hp4</td>
<td>40.39 ± 7.25a</td>
<td>50.42 ± 5.02b</td>
<td>24.93 ± 2.33b</td>
<td>1077.63 ± 144.79d</td>
<td>1193.37</td>
</tr>
<tr>
<td>Hp5</td>
<td>19.23 ± 3.13c</td>
<td>109.16 ± 15.09a</td>
<td>13.31 ± 0.54c</td>
<td>3550.04 ± 193.31a</td>
<td>3691.74</td>
</tr>
<tr>
<td>Hp6</td>
<td>41.75 ± 4.49a</td>
<td>55.95 ± 13.06b</td>
<td>48.95 ± 13.43a</td>
<td>988.50 ± 88.66d</td>
<td>1135.15</td>
</tr>
</tbody>
</table>

Values marked with the same letter do not differ significantly p > 0.05.
antidepressant effects than plants grown in controlled conditions. The most important antioxidant constituents in the samples analyzed were polyphenols with chlorogenic acid as the predominant compound. It may be beneficial to obtain St. John’s wort from natural habitats as they have been found to have higher contents of pseudohypericin and hypericin. Moreover, consuming St. John’s wort in the form of an infusion can provide therapeutic doses of hypericin and other beneficial phytocompounds.

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Conflict of interest: The authors declare no conflict of interest.

Ethics approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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