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RESEARCH PAPER

Variability in the Content of the Constituents of *Hypericum perforatum* L. and Some Commercial Extracts

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ABSTRACT

Seven samples of Hypericum perforatum *L. (St. John's wort) were collected throughout Tuscany; the dried extracts were assayed to determine the concentration of the constituents. Total flavonol content ranged from 4.58% to 15.90%; hypericins ranged from 0.05% to 0.11%; and hyperforins ranged from 1.37% to 20.80%. In addition, four commercially dried extracts were analyzed using the same high-performance liquid chromatographic (HPLC) method; their flavonol contents varied from 10.64% to 15.01%, hypericins varied from 0.03% to 0.20%, and hyperforins varied from 1.18% to 6.54%. The aim of this investigation was to evaluate the contents of the different constituents depending on environmental factors and drying and storage conditions of the wild samples. In addition, the contents of the constituents of the products available to the consumer that were related to quality and the relation of this to safety and efficacy were also evaluated.*

Key Words: *Content variability; Drying and storage conditions; Environmental factor;* Hypericum perforatum *L. (St. John's wort); Naphthodianthrones and phloroglucinols; Wild and commercial samples.*

INTRODUCTION

Hypericum perforatum L., popularly called St. John's wort, has been known since antiquity for its many medicinal properties (1). It is an herbaceous perennial plant, distributed in Europe, Asia, and Northern Africa and naturalized in the United States. In Italy, it is known in all regions and has different trivial names: ''Trascalan'' (Liguria), ''Erba della Madonna'' (Veneto), ''Brunnulidda'' (Sicily), and ''Erba di S. Giovanni'' (most of Italy) (2).

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In the last two decades, anti-inflammatory (3), antimicrobial (4), antiviral (5), and antidepressant (6) activities have also been attributed to the total phytocomplex or single constituents. In Europe, millions of daily doses of preparations containing extracts of *Hypericum perforatum* are used for treating mild to moderately severe depressive disorders and other health conditions, such as anxiety and sleep disorders (7,8). The extract contains a variety of compounds, including quinic acid derivatives, a broad range of flavonols, based on 3-*O*-glucoside of quercetin; naphthodianthrones (hypericin and pseudohypericin); and phloroglucinol derivatives such as hyperforin, adhyperforin, and others, to which great antibiotic effects (9) have been attributed since the 1970s.

For 10 years, antidepressant activity has been related to flavonols and hypericins, but recent pharmacological and clinical studies have pointed out that hyperforin plays an important role in the antidepressant activity of the extracts (10). Thus, the standardization of the extracts based on hypericin ultraviolet (UV) assay can no longer be proposed as a tool to evaluate the potential benefits, or risks, of St. John's wort preparations.

In our investigation, flavonoids, naphthodianthrones, and phloroglucinols were evaluated by high-performance liquid chromatography–diode array detection (HPLC-DAD) and HPLC with mass spectrometry (HPLC-MS) analyses, with special regard to the phloroglucinol content. In general, the biological effects of herbal preparations and their derivatives are considered to arise from the whole mixture of the main principles, but in the case of phloroglucinols, it seems that only hyperforin and adhyperforin should be active, while their oxidized derivatives should be inactive. For this reason, the content of each hyperforinlike constituent is reported.

Seven wild samples of St. John's wort collected in Tuscany (Italy) were evaluated to verify the variations of the content of the constituents depending on different factors. These factors are growth environment, quantities of the different plant parts used (leaf, stem, and/or flowers), harvest time (flowering or fruiting time), drying procedures, and storage conditions (9,11). The results are useful to find the harvest time and the mode of extraction of the phytocomplex to obtain the maximum concentration of the constituents.

In addition, the evaluation of the contents of the constituents was conducted on four commercially dried extracts to determine the amount of the different constituents. The manufacturers are not specifically reported since the purpose of this investigation was not to criticize the quality of the marketed products, but to evaluate the contents of the constituents for different products available to the consumers.

EXPERIMENTAL

Chemicals

Acetonitrile and MeOH HPLC grade, and 85% phosphoric acid were purchased from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

The standard sample of Rutin trihydrate (HPLC potency 96.25%) was kindly supplied by Indena Chemical Laboratories (Settala, Milano, Italy).

Plant Collection and Extraction

All plant samples were collected in Tuscany (central Italy) in summer 1999. Voucher plant samples are maintained in the Department of Pharmaceutical Sciences, University of Florence.

Three plant samples (two consisting of flowering tops and one of fruiting tops) were collected at the beginning of August on a dry and sunny day. They were immediately subjected to extraction to obtain samples A, B, and C. Samples A (flowering tops) and B (fruiting tops) were stored in the dark during extraction. Sample C (flowering tops) was exposed to sunlight during extraction, as suggested by traditional medicine practice.

Two additional samples (one consisting of flowering tops and one of fruiting tops) were collected in late September on a rainy day. The moisture due to rain was removed, and each sample was divided into two parts. One part of each of these samples was extracted as such, without other treatments, to obtain samples D (flowering tops) and F (fruiting tops). The other part of each sample was air dried at room temperature in the dark and, immediately after, was extracted in the same manner as fresh samples to obtain samples E (flowering tops) and G (fruiting tops).

Ground tops of fresh material (150 g) were macerated in 400 ml of methanol. Dried material was treated in the same manner, taking into account the percentage weight loss (67.8% for the flowering tops and 65.8% for the fruiting tops). During extraction (7 days), all samples (with the exception of C) were kept in the dark (bottles were wrapped in aluminum foil) at +5°C. Finally, all the macerates obtained from the extraction of wild plant

material were filtered and concentrated by rotary evaporation (Büchi, Goeppingen, Germany) at room temperature (20°C) under reduced pressure (5 mbar); the samples were shielded from light with the aluminum foil. Finally, the aqueous residues were lyophilized (Lyovac GT Leybold-Heraeus GmBH, Köln, Germany).

Commercial Samples

Four commercially dried extracts of *Hypericum perforatum* L. were kindly offered by Aboca S.p.A. (Sansepolcro, Arezzo, Italy; lot 8J2152); Arkopharma s.r.l. (Largo Olgiata, Roma; Italy; lot 980600056); Specchiasol (San Bonifacio, Verona, Italy, lot 69398); and Indena (Settala, Milano, Italy, lot 26675/M2). Samples obtained from commercial extracts were indicated as H, I, J, and K, randomly coded, and not identified as to source during assay.

Sample Preparation

Methanol solutions of 2.50 mg dried extract per milliliter were prepared. Samples were sonicated for 10 min and filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane (13 mm diameter, 0.45 μm porosity; Lida Manufacturing Corp., WI, USA) before HPLC analysis.

High-Performance Liquid Chromatographic Analysis

Apparatus

The HPLC system consisted of a HP 1090L instrument with a diode array detector and managed by a HP 9000 workstation (Hewlett Packard, Palo Alto, CA). The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett Packard). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. The same column, mobile phase, time period, and flow rate were used. Mass spectrometry operating conditions were optimized to achieve maximum sensitivity values: gas temperature 350°C at a flow rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30°C, and capillary voltage 3500 V. Full scan spectra from *m/z* 100 to 800 in the negative and positive ion modes were obtained (scan time 1 s).

Table 1

Timetable of the High-Performance Liquid Chromatography Program

Time (min)	% H_2O	% CH_3OH	% CH_3CN	Flow (ml/min)
0.0	100.0	0.0	0.0	1.0
10.0	85.0	0.0	15.0	1.0
30.0	70.0	10.0	20.0	1.0
40.0	25.0	10.0	65.0	1.0
55.0	20.0	10.0	70.0	1.0
57.0	5.0	15.0	80.0	1.0
60.0	100.0	0.0	0.0	1.0

Chromatography Conditions

The column was a Protein C4 (5 μm, 250 mm, 0.5 mm id, 300 A; Vydac Separation Group, Hesperia, CA) maintained at 26°C. Chromatographic separation was carried out using three solvents (A = water–85% phosphoric acid [99.7:0.3 v/v]; B = acetonitrile; C = methanol) in a linear gradient program (Table 1). The analysis was for a 50-min period at a flow rate of 1.0 ml/min. Injected volume of the sample was 25 μl solution. Ultraviolet-visible spectra were recorded in the range 200–590 nm, and chromatograms were acquired at 230, 254, 270, 350, and 590 nm.

Calculations

The percentage of constituents in the samples was determined by an external standard quantitation method, comparing areas of the sample peaks with those of the standard, according to the literature data (12). The following equation was applied:

$$\text{Contents } (\%) = A_{\text{sample}} \cdot 100/RF_{\text{std}} \cdot C_{\text{sample}} \cdot RRF$$

where A_{sample} is the peak of the considered constituent in the test solution (area count), $RF_{\text{std}} \cdot$ is the mean response factor of rutin in the reference solutions [Response factor = Area/(Conc.$_{(\text{mg/ml})}$ · purity/100)], C_{sample} is the concentration of the test solution (mg/ml), and RRF is the response factor of the considered constituent relative to rutin (12). The results were calculated by comparing the constituent content to that of dried extract.

RESULTS

In this work, the analysis of the constituents of dried extracts of *Hypericum perforatum* L., obtained by fresh

Table 2

Total Content of Naphthodianthrones and Flavonoids of Samples A–K

Sample	Naphthodianthrones (%)	Flavonoids (%)
A	0.10	9.58
B	0.11	11.45
C	0.11	6.83
D	0.08	15.60
E	0.05	5.64
F	0.08	15.90
G	0.08	4.58
H	0.03	15.01
I	0.20	11.28
J	0.04	10.64
K	0.05	12.72

or air-dried wild plant material and from some commercially dried extracts, and their quantitative evaluation were performed. The content of the three characteristic classes of constituents (i.e., flavonols, naphthodianthrones, and phloroglucinols, paying special attention to naphthodianthrones and especially phloroglucinols, which are unstable for heat and light; 13) are reported in Tables 2 and 3. Some quinic acid derivatives (compounds with retention times of 8.29, 14.49, 19.21, and 39.06 min; Fig. 1) were also identified, but were not evaluated because their influence in the antidepressant activity has not been demonstrated or at least hypothesized. Analyses were performed in triplicate. The content is reported as a percentage of the dried extract.

All the investigated samples showed analogous chromatographic profiles, but some interesting differences among the various extracts were assessed (Fig. 1).

Flavonol (I–VI, Fig. 2) content ranged from 4.58% to 15.90%. There was extreme variability among extracts obtained from wild collection as compared to commercial samples, which ranged from 10.64% to 15.01%. In the extracts obtained from wild samples, the highest values of flavonols were obtained in samples D (15.60%) and F (15.90%), which were obtained from fresh plants (flowering and fruiting tops, respectively) collected at the end of summer. If the same samples were subjected to extraction after drying, the content was reduced to one-third (5.64% and 4.58%, respectively). Low percentages were obtained from plants collected at the beginning of August (9.58% and 11.45%); however, light during extraction caused a degradation of flavonols (from 9.54% to 6.83%).

Naphthodianthrones, including hypericin (VII, Fig. 2) and pseudohypericin (VIII, Fig. 2), ranged from 0.05% to 0.11% in the samples obtained from wild plants. No statistical differences were present in fruiting and flowering tops (0.11% versus 0.10% and 0.08% versus 0.08%, respectively) and between fresh and dried material (0.08% versus 0.05% and 0.08% versus 0.08%). In addition, exposure to sunlight did not determine an increase of hypericin content (from 0.10% to 0.11%). The content of naphthodianthrones of the commercially dried extracts was quite variable (from 0.03% to 0.20%) and generally was very different from the value reported on the label, measured using an UV assay, according to the European Pharmacopoeia (14).

Table 3

Content of the Different Phloroglucinols of Samples A–K

Sample	Furohyperforin (%)	Oxyhyperforin (%)	Hyperforin (%)	Adhyperforin (%)
A	3.66	1.13	10.95	1.41
B	3.95	3.15	10.14	3.56
C	0.30	0.19	0.63	0.25
D	0.85	n.d.	8.25	2.58
E	0.52	n.d.	3.55	1.39
F	1.52	0.18	8.63	4.22
G	0.83	0.25	5.20	2.90
H	0.33	0.23	4.62	1.36
I	0.24	0.34	0.52	0.08
J	0.57	1.03	3.02	0.83
K	0.39	0.05	2.65	1.08

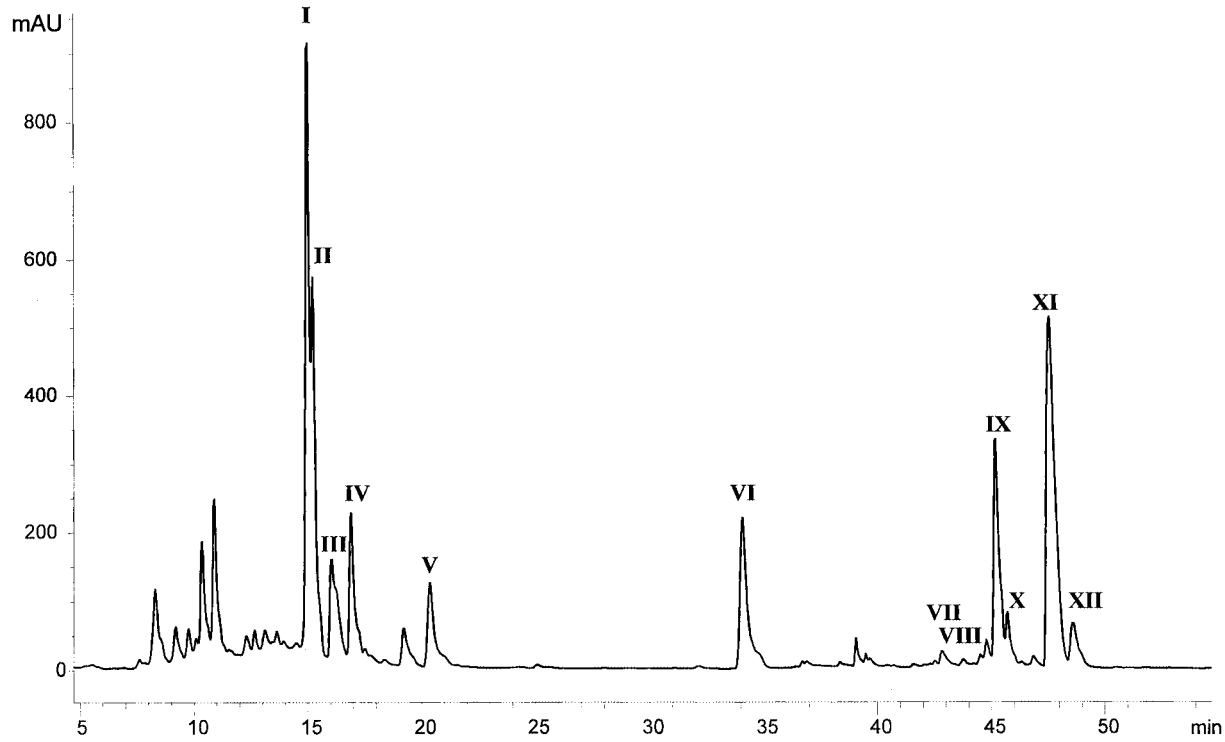

Figure 1. HPLC separation of active constituents (I–XII) of *Hypericum perforatum* L.

Concerning the phloroglucinol content of the marketed extracts, extreme variability of the percentages among the derivatives was evidenced. Hyperforin (XI, Fig. 3) and adhyperforin (XII, Fig. 3), the supposed active constituents, ranged from 0.52% to 4.62% and from 0.08% to 1.36% in extracts H and I, respectively. Also, the extracts obtained from wild samples showed an interesting variability. The highest percentages of these constituents were found in extracts obtained from fresh samples (from 8.24% to 10.95% of hyperforin and from 1.41% to 4.22% of adhyperforin).

Significantly lower amounts of these derivatives were detected in air-dried samples E and G (3.55% and 5.20% for hyperforin and 1.39% and 2.90% for adhyperforin, respectively). The content of sample C obtained by extraction under sunlight was very critical: Hyperforin was 0.63%, and adhyperforin was 0.25%.

Also the content of the other two phloroglucinols, furohyperforin (IX, Fig. 3) and oxyhyperforin (X, Fig. 3), considered as inactive metabolites of the hyperforin was quite varied. In the commercial extracts, their content ranged from 0.24% to 0.57% and from 0.05% to 1.03%, respectively. If we considered the wild samples, the highest amounts of these oxidized metabolites were found in the fresh samples collected in August (3.66% and 3.95% of furohyperforin and 1.13% and 3.15% of oxyhyperforin). If the extraction was performed in sunlight, the content of these two metabolites decreased suddenly (from 3.66% to 0.30% of furohyperforin and from 1.13% to 0.19% of oxyhyperforin). Wild samples collected in September showed lower amounts of both metabolites (0.85% and 1.52% of furohyperforin and at least 0.18% of oxyhyperforin). Similar amounts were found in samples submitted to the drying process.

DISCUSSION

To our knowledge, this is the first report on the comparison of HPLC analysis of all the characteristic constituents of some commercial extracts and extracts from wild samples of *Hypericum perforatum* L. The variability of the constituents in the various extracts is stressed, with special emphasis on the naphthodianthrone and phloroglucinol contents. Thus, the first class of constituents is considered the marker (and evaluated using a UV assay

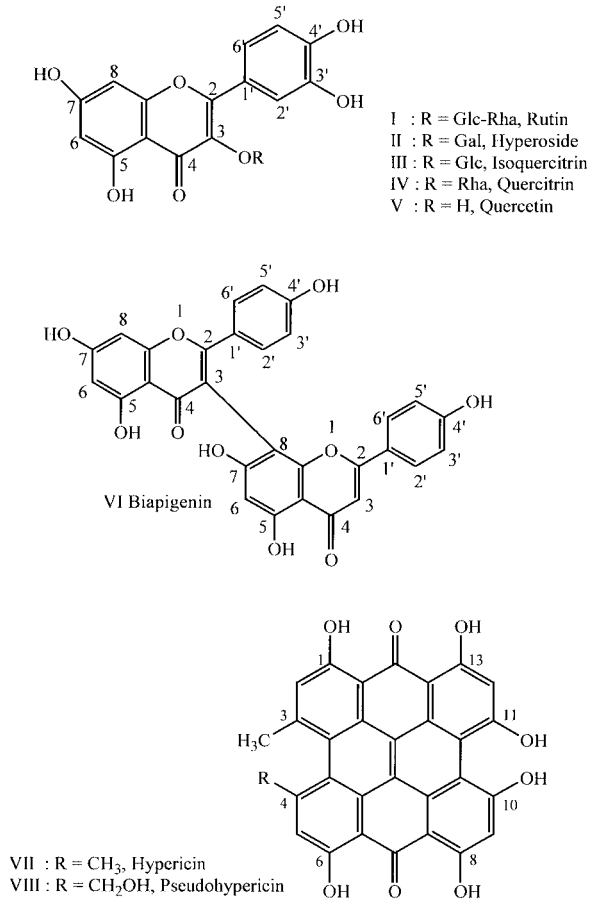

Figure 2. Structures of compounds I–VIII.

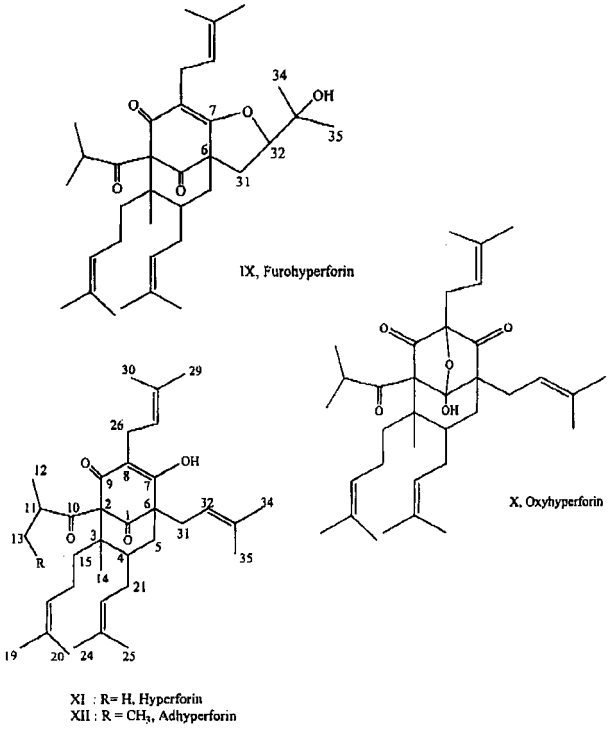

Figure 3. Structures of compounds IX–XII.

by the manufacturers in the standardization of commercial extracts). However, the latter class represents the compounds responsible for the antidepressant activity, and it seems that only hyperforin and adhyperforin should be active, while their oxidized derivatives furohyperforin and oxyhyperforin should be inactive. Therefore, the content of each hyperforinlike constituent was evaluated separately.

Concerning our results, the hypericin content among the marketed extracts was similar, with the exception of product I, for which the content was four- to sevenfold more than the others.

The hypericin content of wild samples was very similar, with a maximum value of 0.11% in samples B and C and a minimum value in sample E.

These results pointed out that hypericin content is probably not correlated to different development stages, parts of plant used, drying process, or exposition to light, as previously reported (9), but probably is correlated to the different solvents used for the extraction and/or the mode of the extraction.

Regarding hyperforinlike constituent contents, we found extreme variability both in the hyperforin and adhyperforin content and in the oxidized metabolites.

In the marketed extracts, the highest value of hyperforin and adhyperforin was found in sample H and the lowest in sample I, while the percentage of the oxidized metabolites was similar in both samples.

Lower content of hyperforin and adhyperforin (3.73% and 3.85%) was found in J and K, respectively, but the percentage of oxidized metabolites in J (1.57%) was threefold that of K (0.44%).

Higher contents of hyperforin and adhyperforin were found in all wild samples submitted to extraction without the drying process. It seems that the development stage and weather conditions do not affect the content of these constituents even if a higher content of oxidized metabolites in samples collected in August (3.66% and 3.95% versus 0.85% and 1.52%, respectively) was evidenced.

Drying process only partly affected the content of hyperforin and adhyperforin (from 10.82% to 4.94% and from 12.85% to 8.10%, respectively); sunlight exposition during the extraction process markedly affected the con-

tent of hyperforin and adhyperforin (from 12.36% to 0.88%). From our results, sunlight exposition is the main condition to be avoided.

In conclusion, we want to remark that extreme variability of the three classes of constituents was found among wild samples and also among samples from commercial manufacturers. Our findings suggest the necessity of the quality control of herbal medicinal products with specific methods, especially if active compounds are not identified, as in the case of St. John's wort, because the concept of quality is fundamental to ensure safety and efficacy of these preparations. Especially for these herbal drugs and their preparations, only HPLC fingerprints can provide an overview of their quality; thus, all classes of constituents can be correctly quantified.

It is clear that, in the case of Saint John's wort extracts and their preparations, a standardization based on hypericin content, as suggested by the European Pharmacopoeia (14), might not offer a guarantee of pharmacological equivalence.

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