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Analysis and stability of the constituents of St. John's wort oils prepared with different methods

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Abstract

St. John's wort is a medicinal plant with a long history of use in traditional medicine all over Europe. Traditional preparations and in particular the infused oil from SJW flowers remains one of the most popular and curative topical remedy against ulcerations and burns. The presence of the characteristic polyprenylated acylphloroglucinol derivatives, namely hyperforin and analogs are instead related to the oil's therapeutic activity. Indeed, it is well known that hyperforin has a potent antibacterial activity.

In this study we tried to rationalize the production system of the oily preparation in order to obtain the highest concentration and stability of phloroglucinols. Five different samples of SJW oils were evaluated by HPLC-DAD-MS analysis to verify the variability and stability of the constituents according to the following factors: different harvesting time, fresh or dried plant material, use of sunlight or heating systems during extraction. The stability of these oils during 1 year was also tested.
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Keywords: St. John's wort oil; Harvesting time; Extraction procedures; Content variability; Stability

1. Introduction

Hypericum perforatum L. (St. John's wort, SJW) is one of the most interesting medicinal plants with a long history of use in traditional medicine all over Europe; principally for external use in the treatment of burns, bruises, swelling and shingles [1–4]. In the last 20 years standardised alcoholic (60% ethanol or 80% methanol) extracts have become increasingly popular in Europe and the US for the successful treatment of mild to moderate depressive disorders [5–10]. However, traditional preparations and in particular the infused oil from St. John's wort flowers obtained by macerating the flowering tops collected around St. John the Baptist's day (24th June) in olive oil and then exposed to sunlight for 2 or 3 weeks [11] remains one of the most popular and curative topical remedies in Europe against ulcerations and burns. Only a few data were reported about clinical use,

but many pharmacological studies emphasized the vulnerary and epithelising properties [12–15]. In a recent publication [16] the tissue regenerating action of a mixture of oily extracts of St. John's wort and calendula was demonstrated on surgical wounds from childbirth with cesarean section in a group of 24 patients.

Regarding the analysis of traditional Oleum hyperici, it is known that it does not contain hypericin, but the characteristic red colour and fluorescence can be ascribed to lipophilic constituents derived from the degradation of hypericin due to exposure to sunlight [3]. The presence of the characteristic polyprenylated acylphloroglucinol derivatives, namely hyperforin and analogs are instead related to the oil's therapeutic activity, while other two phloroglucinols, namely furohyperforin and oxyhyperforin, are considered as inactive metabolites of the hyperforin [17].

It is well known that hyperforin has a potent antibacterial activity [18]: it can inhibit the growth of Gram-positive bacteria such as *Corynebacterium diphtheriae* [19] and *Staphylococcus aureus* [20,21]. In addition, the pronounced

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antiinflammatory activity of hyperforin may provide a rationale for the topical treatment for inflammatory skin disorders [13,22].

The aim of the present investigation was to rationalize the production system of the oily preparation in order to obtain the highest concentration and stability of phloroglucinols. Five different samples of St. John's wort oils were evaluated to verify the variability and stability of the constituents according to the following factors: different harvesting time (inflorescences collected during flowering or when the fruits are stipulated), fresh or dried plant material, use of sunlight or heating systems during extraction. The stability of these oils during 1 year was also tested.

2. Materials and methods

2.1. Materials

2.1.1. Solvents and reagents

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

Indena Research Laboratories (Settala, Milan, Italy) kindly provided the reference rutin trihydrate (batch no. K12408717, standard purity 96.25% considering the content of residual solvents, moisture and amount of impurities). Olive oil was according to Italian Pharmacopoea (ditta Polichimica S.r.l. Bologna).

2.1.2. Plant materials

Hypericum perforatum L. was a wild sample collected at locality Fonte Grillo (Bassano Romana. Lazio, Italy) 24th June 2005 (inflorescences collected during flowering), and at the beginning of July 2005 (inflorescences collected when the fruits were completely stipulated). Plant material was identified by one of the authors (F. Carnevali) and a voucher specimen of each sample was deposited at ENEA, Dep. BIOTEC-AGRO.

2.2. Methods

2.2.1. Preparation of MeOH extracts

Part of the collected plant material was dried at $50\,°C$ and used for the preparation of the oils or for the HPLC analysis. The percentage weight loss of inflorescences collected during flowering was 66.0%, the percentage weight loss of inflorescences collected when the fruits were completely stipulated was 64.1%. For their quail-quantitative analysis, 50 g of each sample were submitted to exhaustive extraction with MeOH at room temperature under stirring, in the absence of light and for 3 days. The solutions were then filtered and evaporated using a rotary evaporator under vacuum until dryness and the extracts were prepared for HPLC-DAD-MS analyses, as reported in Section 2.2.4 (Table 1).

Table 1
HPLC mobile phase

Time (min)	H_2O/HCOOH (%) (pH 3.2)	CH_3CN (%)	MeOH (%)
0.1	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	10	80	10
60	100	0	0

2.2.2. Preparation of the oils

Sample 1: 280 g fresh St. John's wort flowers macerated for 28 days with 1.6 l of olive oil and exposed to sunlight during extraction.

Sample 2: 30 g dried St. John's wort flowers macerated for 28 days with 570 ml of olive oil and exposed to sunlight during extraction.

Sample 3: 30 g dried St. John's wort flowers macerated for 3 days with 570 ml of olive oil at $50\,°C$ in the absence of light.

Sample 4: 90 g fresh St. John's wort fruits (capsules) macerated for 28 days with 512 ml of olive oil and exposed to sunlight during extraction.

Sample 5: 90 g fresh St. John's wort fruits (capsules) macerated for 3 days with 512 ml of olive oil at $50\,°C$ and in presence of artificial light.

2.2.3. Stability study

After the preparation, all the oils were shielded from light using dark bottles and stored at room temperature ($25\,°C$). Starting from November 2005 (about 4 months after their preparation), a sample of each oil was collected monthly and analyzed by HPLC/DAD/MS to evaluate the stability of constituents.

2.2.4. Preparations of the samples for HPLC/DAD/MS analyses

The samples were prepared and immediately injected. 5 mg MeOH extract was exactly weighed and solubilised in 1 ml MeOH before the analysis. All the oil samples were exactly weighed (0.5 g) and a mixture of chloroform:methanol 4:6 was added (10 ml). All the samples were filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (13 mm diameter; 0.45 μm porosity, Lida Manufactoring Corp., WI, USA), before HPLC analysis.

2.2.5. HPLC-DAD and HPLC-MS drug analyses

The HPLC system consisted of a HP 1100L instrument with a diode array detector and managed by a HP – Chem workstation (Agilent Technologies, Palo Alto, CA, USA). The reverse-phase column was a 201 TP 54 (5 μm, 250 mm, 0.5 mm i.d., 300 Å, Vydac Separation Group Hesperia, CA, USA) maintained at $26\,°C$. The mobile phase was a five-step linear solvent gradient CH_3CN/CH_3OH/H_2O (pH 3.2, HCOOH) over a 60 min period at a flow rate of 1 ml/min. The mobile phase is reported in Table 1. The injected volume of sample was a 20-μl solution. UV–vis spectra were recorded in the range 200–590 nm,

and chromatograms were acquired at 230, 254, 270, 350 and 590 nm.

The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). 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 the HPLC-DAD analysis. The same column, mobile phase, time period and flow rate were used. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values; gas temperature 350 °C at a flow rate of 10 l/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from m/z 100–800 in the positive ion mode were obtained (scan time 1 s).

2.2.6. Quantitative analysis

To evaluate the constituent content, a HPLC analysis was performed using a method described in the literature [23], modified for our experimental necessities. Quantification of the constituents was performed using rutin as an external standard and consideration of each constituent and the relative response factor (RRF) with respect to the rutin, as previously reported [23]. The following equation was applied:

$$\text{Content\%} = \frac{A_{\text{Sample}} \times 100}{\text{RF}_{\text{std}} \times \text{Conc}_{\text{sample}} \times \text{RRF}}$$

where A_{sample} is the peak of the considered constituent in the test solution (area count), RF_{std} the mean response factor of rutin in the reference solutions [response factor = area × 100/($\text{Conc}_{(\text{mg/ml})}$ purity)] C_{sample} is the concentration of the test solution (mg/ml), and RRF is the response factor of the considered constituent relative to rutin [23]. The results were calculated by comparing the constituent content to that of dried extract.

3. Results

Different preparation procedures of Oleum hyperici – namely different harvesting time, fresh or dried plant material, use of sunlight or heating systems during extraction – were evaluated in order to clarify the qualitative and quantitative composition of constituents of this popular preparation.

As the first step in this investigation the content of the MeOH extracts of the different herbal samples (inflorescences collected during flowering or when the fruits were stipulated) were evaluated in triplicate by HPLC-DAD-MS analysis and reported as a percentage (Table 2), referring to the dried plant material.

The three characteristic classes of constituents, i.e. flavonoids, naphthodianthrones and phloroglucinols were quantified, paying special attention to naphthodianthrones and especially phloroglucinols which are unstable in the presence of heat and light [3], while the quinic acid derivatives were identified but not evaluated because their possible influence in activity of the preparation has not been demonstrated.

The HPLC profile of MeOH extract is reported in Fig. 1. The oils showed an analogous chromatographic profile. Some quinic acid derivatives (compounds with retention time below 15 min)

Table 2
Content (%) of the different constituents found in dried samples of SJW inflorescences collected during flowering (FL) or collected when the fruits are completely stipulated (FR)

	Phloroglucinols			
	Oxihyperforin	Furohyperforin	Hyperforin	Adhyperforin
FL	0.438 ± 0.040	0.224 ± 0.014	3.151 ± 0.026	0.479 ± 0.031
FR	0.709 ± 0.030	0.278 ± 0.019	3.969 ± 0.020	0.741 ± 0.035
	Flavonoids			
	Rutin	Hyperoside	Quercitrin	I3,II8-Biapigenin
FL	0.753 ± 0.020	0.493 ± 0.012	0.255 ± 0.009	0.333 ± 0.011
FR	0.910 ± 0.022	0.534 ± 0.014	0.262 ± 0.008	0.433 ± 0.010
	Naphtodianthrones			
	Pseudohypericin			Hypericin
FL	0.069 ± 0.003			0.039 ± 0.002
FR	0.035 ± 0.002			0.014 ± 0.001

were also identified in both the samples on the basis of their UV and MS data but were not evaluated because their influence in the activity of SJW oil has not been demonstrated or at least hypothesised.

Total flavonoid (I–VI, Fig. 1) content of SJW inflorescences collected during flowering or when the fruits were completely stipulated (FR) was similar and ranged from 1.834 to 2.139%, with the highest value found in FR (Table 2). The proportional ratio among the different constituents was very similar.

Naphthodianthrones, including hypericin (VIII, Fig. 1) and pseudohypericin (VII, Fig. 1) had a different percentage in fruit-
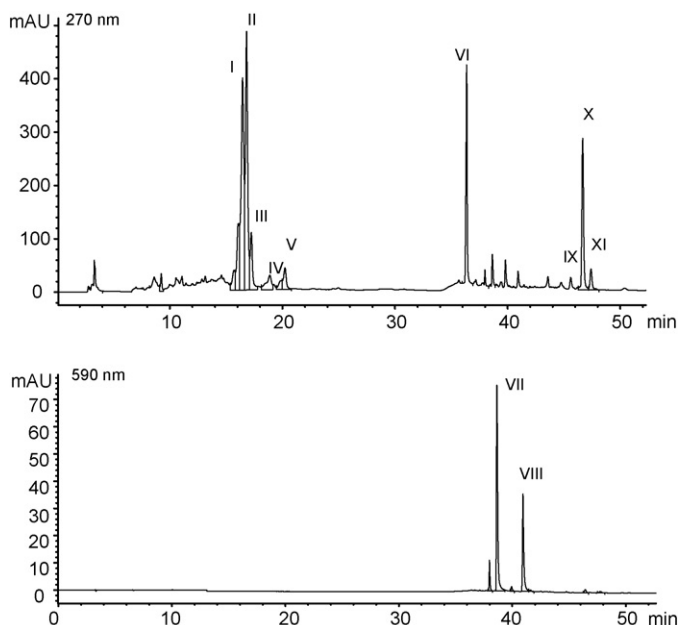

Fig. 1. HPLC profile at 270 nm and 590 nm of *Hypericum perforatum* L. 270 nm: t_R = 16.45 min, rutin I; t_R = 16.80 min, hyperoside II; t_R = 17.23 min, isoquercitrin III; t_R = 18.87 min, quercitrin IV; t_R = 20.21 min, quercetin V; t_R = 36.35 min, I3, II8-biapigenin VI; t_R = 45.60 min, furohyperforin IX; t_R = 46.66 min, hyperforin X; t_R = 47.40 min, adhyperforin XI. 590 nm: t_R = 38.63 min, pseudohypericin VII; t_R = 40.93 min, hypericin VIII.

Table 3
Content (μg/100 mg oil) and extractability percentage (%E) of the different constituents found in Samples 1–5

	Flavonoids		Phloroglucinols				Furohyperforin
	I3,II8-Biapigenin		Hyperforin		Adhyperforin		
	μg/100 mg	%E	μg/100 mg	%E	μg/100 mg	%E	μg/100 mg
Sample 1	3.51 ± 0.74	18.71	26.58 ± 1.96	14.97	8.52 ± 1.30	31.58	n.d.
Sample 2	n.d.	–	16.89 ± 1.62	10.18	2.50 ± 1.12	9.92	n.d.
Sample 3	0.44 ± 0.01	2.51	6.21 ± 0.85	3.74	1.18 ± 0.02	4.68	12.63 ± 1.53
Sample 4	n.d.	–	36.90 ± 1.67	19.48	17.58 ± 1.09	39.45	n.d.
Sample 5	0.35 ± 0.06	1.34	4.50 ± 0.68	2.38	10.17 ± 0.83	22.82	n.d.

Table 4a
Stability of the constituents of sample 1 (280 g fresh St. John's wort flowers macerated for 1 month with 1.6 l of olive oil and exposed to sunlight during extraction)

Months of storage	Phloroglucinols content (μg/100 mg oil)		Flavonoids content (μg/100 mg oil)
	Hyperforin	Adhyperforin	I3,II8-Biapigenin
Time zero	26.58 ± 1.96	8.52 ± 1.30	3.51 ± 0.74
4	11.07 ± 1.47	1.04 ± 0.51	3.17 ± 0.34
5	8.12 ± 0.30	0.77 ± 0.34	2.47 ± 0.18
6	9.10 ± 1.02	0.65 ± 0.26	2.43 ± 0.24
8	9.17 ± 0.47	0.72 ± 0.23	2.74 ± 0.20
12	8.07 ± 0.73	0.61 ± 0.21	2.57 ± 0.14

ing and flowering tops (0.014% versus 0.039%, and 0.035% versus 0.069%) and the hypericin value was generally an half value of pseudohypericin (Table 2).

Concerning the phloroglucinol content, the highest percentages of the native constituents, namely hyperforin and adhyperforin, were found in the fruiting tops (3.969 and 0.741%, respectively). The other two phloroglucinols, namely furohyperforin (IX, Fig. 1) and oxyhyperforin (X, Fig. 1), considered as inactive metabolites of the hyperforin, were mostly present in the fruiting tops (0.278 and 0.709%, respectively, Table 2).

Analyses were then carried out on the different oils immediately after their preparation by HPLC-DAD-MS and results (μg constituent/100 mg preparation) are reported in Table 3. All the injections were performed in triplicate. All the oils did not contained hypericin-like constituents, as previously reported in the literature [3,12]; also the flavonoids were not present with the exception of I3,II8-biapigenin.

Concerning the phloroglucinols, extreme variability of the percentages among the derivatives was found (Table 3). Hyperforin ranged from 369.0 to 45.0 ppm, in the samples 4 and 5, respectively; adhyperforin ranged from 175.8 to 11.8 ppm, in the

samples 4 and 3, respectively. The highest percentages of these constituents were found in sample 4 obtained from fresh capsules and biapigenin was not present in this oil. Sample 3 showed very low values of all the tested constituents and it represented the only sample where furohyperforin, an inactive metabolite of the hyperforin, was found (126.3 ppm). The extraction performed in oven at 50 °C (samples 3 and 5) reduced the quantities of phloroglucinols in the final product.

As a second part of this work, the stability of the oils over a 1-year period was tested by HPLC-DAD-MS analysis. After the preparation, 10 ml of each oil were shielded from light with aluminium foil and kept in the freezer (−20 °C) for the HPLC analysis (time 0). Subsequently, the HPLC analysis of the oils was carried out monthly to test the thermal stability testing. The results of stability testing, during the 12 months' storage are reported in Tables 4a–4e.

After such period, the residual content of hyperforin was a third of the initial value both in samples 1 and 4. In the oils prepared using dried material (samples 2 and 3) and having the lower percentages of hyperforin the degradation was complete (sample 2) or about 90% (sample 3) after 1 year. In sample 5 the

Table 4b
Stability of the constituents of sample 2 (30 g dried St. John's wort flowers macerated for 1 month with 570 ml of olive oil and exposed to sunlight during extraction)

Months of storage	Phloroglucinols content (μg/100 mg oil)		Flavonoids content (μg/100 mg oil)
	Hyperforin	Adhyperforin	I3,II8-Biapigenin
Time zero	16.89 ± 1.62	2.50 ± 1.12	n.d.
4	3.58 ± 0.92	3.41 ± 0.74	n.d.
5	1.44 ± 0.39	n.d.	n.d.
6	n.d.	n.d.	n.d.
8	n.d.	n.d.	n.d.
12	n.d.	n.d.	n.d.

Table 4c
Stability of the constituents of sample 3 (30 g dried St. John's wort flowers macerated for 3 days with 570 ml of olive oil at 50 °C in the absence of light)

Months of storage	Phloroglucinols content (μg/100 mg oil)			Flavonoids content (μg/100 mg oil)
	FuroHyperforin	Hyperforin	Adhyperforin	I3,II8-Biapigenin
Time zero	12.63 ± 1.53	6.21 ± 0.85	1.18 ± 0.02	0.44 ± 0.01
4	15.38 ± 0.88	1.64 ± 0.33	n.d.	0.17 ± 0.01
5	9.44 ± 0.68	0.84 ± 0.30	n.d.	n.d.
6	15.71 ± 1.27	0.81 ± 0.03	n.d.	n.d.
8	14.26 ± 0.60	0.83 ± 0.11	n.d.	n.d.
12	13.65 ± 0.05	0.80 ± 0.05	n.d.	n.d.

Table 4d
Stability of the constituents of sample 4 (90 g fresh St. John's wort fruits (capsules) macerated for 1 month with 512 ml of olive oil and exposed to sunlight during extraction)

Months of storage	Phloroglucinols content (μg/100 mg oil)		Flavonoids content (μg/100 mg oil)
	Hyperforin	Adhyperforin	I3,II8-Biapigenin
Time zero	36.90 ± 1.67	17.58 ± 1.09	n.d.
4	16.22 ± 1.19	n.d.	n.d.
5	12.49 ± 0.63	n.d.	n.d.
6	13.06 ± 1.19	n.d.	0.24 ± 0.02
8	14.17 ± 0.42	n.d.	0.22 ± 0.04
12	13.22 ± 0.53	4.52 ± 0.53	0.21 ± 0.03

residual content of hyperforin (21.9 ppm) was about an half of the initial value (45.0 ppm).

Adhyperforin degraded very quickly and after 1 year it was still present in only one sample (sample 1, 6.1 ppm); in all the other oils it was no longer detectable after this period. In the case of sample 3, the only sample which evidenced the presence of furohyperforin, this constituent had a similar content over time, probably deriving from the degradation of hyperforin.

Biapigenin was sufficiently stable in sample 1 and after 1 year 73% of the starting value was still present. No other samples showed the presence of this biflavonoid.

4. Discussion

Oleum Hyperici is a traditional preparation for topical application obtained from maceration of the flowering tops with olive oil. This paper reports a comparison of HPLC analysis of the constituents of Oleum Hyperici prepared by traditional method (DAB 6) and other procedures to find the best condition of extraction of the phytocomplex in order to obtain a maximum concentration of constituents.

Several oils were prepared according to different factors: different harvesting time, namely inflorescences collected during flowering or when the fruits were stipulated, use of fresh or dried herbal drug, heating system (sunlight or oven). As evidenced by results reported in Tables 2 and 3, only some constituents can be extracted during maceration and their variability, especially phloroglucinol content, to which the bactericidal and antiinflammatory activity of oil is attributed, in the various oils is considerable. Extractability of hyperforin ranged from 19.48 to 2.39% and the maximum extraction was obtained with fresh plant material exposed to sunlight during maceration (samples 1 and 4). The drying process and use of artificial light during maceration affected the extraction of hyperforin, producing very low percentages of this compound (samples 2, 3 and 5). Adhyperforin showed a better extractability when fresh plant material was used (31.58% for sample 1, 39.45% for sample 4, and 22.82% for sample 5). Furohyperforin, an oxidated product of hyperforin, was identified only in sample 3 (12%), probably derived from the modification of the phoroglucinol with heat.

Neither naphthodianthrones nor flavonoids, with the exception of I3, II8-biapigenin, were found in the oils. However, its

Table 4e
Stability of the constituents of sample 5 (90 g fresh St. John's wort fruits (capsules) macerated for 3 days with 512 ml of olive oil at 50 °C and in presence of artificial light)

Months of storage	Phloroglucinols content (μg/100 mg oil)		Flavonoids content (μg/100 mg oil)
	Hyperforin	Adhyperforin	I3,II8-Biapigenin
Time zero	4.50 ± 0.68	10.17 ± 0.83	0.35 ± 0.06
4	1.97 ± 0.06	2.04 ± 0.41	0.16 ± 0.02
5	2.07 ± 0.11	1.03 ± 0.14	0.16 ± 0.02
6	2.03 ± 0.05	n.d.	n.d.
8	2.06 ± 0.06	n.d.	n.d.
12	2.19 ± 0.17	n.d.	n.d.

content had a wide variability: the highest percentage was found in sample 1 (35.1 ppm), while it was not detected in samples 2 and 5, stressed with heat and/or light.

Concerning the stability study during 12 months' storage, the results evidenced that the traditional oil (sample 1) and the oil obtained by extraction of fresh capsules (sample 4) represent the best procedures to obtain high phloroglucinol content, which is responsible for the oil's activity.

5. Conclusions

During preparation of Oleum Hyperici only some constituents can be extracted during maceration and their variability, especially hyperforin and adhyperforin, to which the bactericidal and antiinflammatory activity of oil is attributed, in the various oils is considerable. The maximum extraction of the hyperforin was obtained with fresh plant material exposed to sunlight during maceration. The drying process and use of artificial light during maceration affected the extraction of hyperforin, producing very low percentages of this compound. Adhyperforin showed a better extractability from fresh plant material. Neither naphthodianthrones nor flavonoids, with the exception of biapigenin, were found in the oils, however, its content had a wide variability and it was not detected when the oil was stressed with heat and/or light.

Concerning the stability study during 12 months' storage, the results evidenced that the traditional oil and the oil obtained by extraction of fresh capsules represent the best procedures to obtain high phloroglucinol content.

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