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Investigation of impact of storage conditions on *Hypericum perforatum* L. dried total extract



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ABSTRACT

Hypericum perforatum L. (Hypericaceae) has been widely prescribed for mild to moderate depression following the release of promising results in clinical trials. However, it is known that its constituents may be affected by milieu. The stability complexities of the constituents of *H. perforatum* have gained interest in recent years. The aim of the present study was to examine the impact of storage conditions on *H. perforatum* total extract simultaneously under different storage conditions. Temperature, humidity, and light conditions were evaluated. Comparative analyses of methanol extracts were conducted using high performance liquid chromatography-diode array detection for chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, pseudohypericin, hyperforin, and hypericin. Analysis and extraction were performed using a validated method. The fluctuation of the constituents of the plant extract has been demonstrated. Among these components, chlorogenic acid was the most stable. Hyperforin, hypericin, and pseudohypericin were more stable than the flavonoids at -20°C , in the 6th month. As estimated, decay was lowest at -20°C and highest at 40°C –75% relative humidity for the analyzed constituents. Except for hyperforin, light protection decreased the breakdown of components within 4 months. However, at the 6th month, equivalent changes were seen for all constituents. Degradation of the constituents at -20°C indicates the importance of stability tests in analysis studies covering time and storage conditions.

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1. Introduction

Plants and their preparations have been used by humans in the treatment of various conditions for thousands of years. Use of traditional medicine including medicinal plants remains widespread in developing countries, whereas

complementary and alternative medicine use continue to thrive in developed countries [1]. Investigations support complementary and alternative medicine for physical and psychiatric disorders [2]. Thus, medicinal plants are still being widely studied in contemporary pharmaceutical sciences.

Hypericum perforatum L. (HP) has been used for centuries. Its efficacy and indications of use have been well documented by

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numerous scientists beginning with Hippocrates (400 B.C.), followed by Paracelsus [3], and today it is compiled in various pharmacopeias such as European Pharmacopeia, European Scientific Cooperative on Phytotherapy (ESCOP) European Medicines Agency, and World Health Organization Monographs. Commonly, HP has been indicated internally as an antidepressant and used as a wound healer agent externally. Popular products based on this plant, which are used for the treatment of mild and moderate depression, account for a substantial market share in the United States; additionally, it has been prescribed more often than fluoxetine HCl in Germany [4]. HP is a plant tested in numerous clinical trials [5], providing a high level of evidence results [6], whereas it is classified as the most commonly used species in ethnobotanical surveys [7]. In the search for new compounds, quantification of constituents, extract optimization, and standardization of *Hypericum* species is an important topic [8–11].

Stability tests have been an important part of the testing program for both drug substances and herbal preparations. Recently, investigations on the stability of commonly used herbal extracts have drawn increasing attention [12–15]. Stability is an important issue for HP, which has complex ingredients: hypericin, pseudohypericin (naphthodianthrones); hyperforin (phloroglucinol); hyperoside, quercitrin, quercetin, rutin (flavonoids); and chlorogenic acid (phenolic acids) [16]. Previous reports have demonstrated some problems in relation to the stability of HP extracts. In a study, interdays stability and effects of filtration on major constituents of methanol extract were determined [17]. In another study, hyperforin stability was studied on the lipophilic fraction of extract analyzed using high-performance liquid chromatography (HPLC)–mass spectrometry (MS) [18]. The stability of HP oils prepared with different methods was analyzed using the HPLC–diode array detection (DAD)–MS system [13]. Flavonoids, naphthodianthrones, and phloroglucinol derivatives were evaluated in tincture with HPLC–DAD–MS for accelerated and long-term testing [12]. The stability of hyperforin in maceration of HP dried flowers was also determined using the HPLC system [19]. The thermal and photostability of commercial dried extract was investigated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) testing conditions [16]. Marketed formulations were analyzed for their hypericin and pseudohypericin content concerning temperature and humidity [20]. The relation between physical and chemical characteristics was likewise examined for HP products [21]. The stability of hypericin and pseudohypericin in extracts of *Hyperici herba* and standard solutions were studied under different temperatures and light conditions; the results were monitored with visible spectroscopy and HPLC–VIS/DAD [22]. In another study, the content uniformity of the plant and batch-to-batch reproducibility in HP products were investigated [23]. All these studies have important contributions to what we know about extract stability. However, the stability of dried total extract still has complexity and requires further clarification in terms of gathering conditions in one investigation concerning storage parameters such as light, humidity, and temperature together in parallel control. Therefore, the studied parameters were chosen to simulate the conditions usually encountered such as the effects of light, preservation capability

of cold storage, and the effects of heat and humidity at rough levels to observe the degradation according to ICH guidelines. To the best of our knowledge, this is the first investigation to cover all the major constituents regarding the impact of storage conditions on *H. perforatum* dried total extract, including such parameters as humidity, light, and temperature for 6 months in the same context.

In this study, our aim was to determine the impact of storage conditions—such as temperature, humidity, and light conditions—on the constituents of *Hypericum perforatum* methanolic extract (HPME). Analyses were carried out using the HPLC–DAD system for these components.

2. Methods

2.1. General

HPLC-grade water was obtained with Millipore Type I Ultrapure Water Systems (Millipore, Billerica, MA, USA). HPLC-grade acetic acid was purchased from J.T. Baker (Center Valley, PA, USA); HPLC-grade acetonitrile and methanol were purchased from Labscan (Gliwice, Poland). HPLC standards as hypericin and pseudohypericin were obtained from Planta Natural Products (Vienna, Austria). Rutin, quercitrin, quercetin, and amentoflavone standards were kindly sent by Professor Hasan Kırmızıbekmez (Yeditepe University, Istanbul, Turkey); hyperoside and isoquercitrin standards were kindly given by Professor Emrah Kılınç (Ege University, Izmir, Turkey), and hyperforin standard was kindly supplied by Professor Athanassios Giannis (Leipzig University, Leipzig, Germany).

2.2. Plant material

The plant material, collected from a single population of cultivated plants, was kindly provided by the Faculty of Agriculture, Ege University. The plant was identified at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Ege University, and specimen vouchers are kept at the IZEF Herbarium, (Ege University Faculty of Pharmacy International Herbarium) (No. 5796).

2.3. Extraction method

Air-dried aerial parts of the plant (200 mg) were homogenized using a blender and were extracted with 10 mL of methanol by sonication at 11–13°C for 30 minutes and at 21–23°C for 30 minutes with exclusion of light. The samples were centrifuged at 7000g for 10 minutes. The supernatant was separated, and the same procedure was repeated three more times for the precipitate according to Li and Fitzloff's [17] extraction method. Dry total extract was obtained via evaporation of total solvent using a vacuum evaporator. Total dryness of the extract was achieved using a vacuum concentrator system (yield: 26.5%, w/w).

2.4. Validation of extraction

Validation tests of blank extraction (an extraction procedure done without plant material), spiked blank extraction (blank

extraction with addition of quercetin standard without plant material), reproducibility, repeatability, consistency, and homogeneity (homogeneity of dry extracts in sample tubes from five different points of the tubes) were performed. The recovery test was performed with Morin standard, which could be identified with the same analysis method.

2.5. Storage conditions

Storage conditions were determined as follows: room condition (climatized condition at 25°C with uncontrolled humidity) with daylight (case 1) and room condition without daylight, dark (case 2); 25°C–65% relative humidity (RH; case 3) and 40°C–75% RH (case 4); and –20°C (case 5) and 4°C (case 6; Table 1). For all conditions, three sets of dry extract samples in glass vials were located and analyzed by HPLC ($n = 3$) by preparing fresh samples in each period. The effects of light, temperature, and humidity were studied monthly for 6 months according to the ICH Guidelines [24]. The impact of storage conditions was compared within light conditions, cold storage environment, and ICH guidelines criteria.

2.6. Analysis method

Analyses were performed using the method described by Brolis et al [25], with minor modifications. Analyses were conducted using an Agilent 1100 HPLC system equipped with degasser, autosampler, column oven, gradient pump, and PDA detector (Agilent Technologies, Waldbronn, Germany). A column with 4.6 mm diameter, 250 mm length, and 5 μm C18 particle size (ACE-121-2546) is used with 1 cm guard column 5C18 (HI-5C18-10C). The injection volume was 20 μL , and the flow rate was 1 mL/min. The column oven was set at 30°C; the wavelength was set at 270 nm and 590 nm. The gradient system of the mobile phase is given in Table 2.

The calibration was studied with seven concentration points of five injections for chlorogenic acid (1.25–80 $\mu\text{g/mL}$), rutin (1–64 $\mu\text{g/mL}$), hyperoside (2.5–160 $\mu\text{g/mL}$), isoquercitrin (1.25–80 $\mu\text{g/mL}$), quercitrin (0.5–32 $\mu\text{g/mL}$), quercetin (0.25–16 $\mu\text{g/mL}$), amentoflavone (0.125–8 $\mu\text{g/mL}$), pseudohypericin (0.25–16 $\mu\text{g/mL}$), hypericin (0.25–16 $\mu\text{g/mL}$), and hyperforin (2.5–160 $\mu\text{g/mL}$) with regression (r^2) of > 0.9994.

3. Results and discussion

H. perforatum L. has been widely prescribed following the release of promising results in clinical trials [4]. Consequently,

Table 1 – Storage conditions.

Case	Condition	Aim of investigation
1	25°C—daylight	Effect of light
2	25°C—dark	
3	25°C–65% RH	Effect of temperature and humidity
4	40°C–75% RH	
5	–20°C	Effect of cold storage
6	4°C	

RH = relative humidity.

Table 2 – Gradient analysis system.

Mobile phase (minute)	0	10	30	40	55	56	65
99.7% water–0.3% acetic acid	100	85	70	10	5	100	100
Acetonitrile	0	15	20	75	80	0	0
Methanol	0	0	10	15	15	0	0

the stability complexity of HP compounds has attracted great interest. In this study, HPME constituents were evaluated for the interpretation of stability considering different storage conditions including the effect of light, cold storage conditions, and ICH guidelines criteria.

Validation tests on extraction and analysis were performed. Tests of blank and quercetin spiked blank extraction continued with recovery test. Morin as an external compound similar to analyzed constituents was used for recovery tests, achieving 90% recovery. Extraction repeatability tests gave the following relative standard deviation (RSD) values: chlorogenic acid, 2.1%; rutin, 4.6%; hyperoside, 2.3%; isoquercitrin, 3.8%; quercitrin, 7.8%; quercetin, 14%; amentoflavone, 17.8%; pseudohypericin, 16%; hyperforin, 3.4%; and hypericin, 15.3%. The homogeneity of the dry extract was also tested; considering the part taken from the sample tube that was analyzed could affect the results. Samples from five different points of a dry extract in a test tube were analyzed, and results showed that hyperforin had 10.6% RSD and hypericin had 11.0% RSD, whereas the maximum RSD was observed for quercetin at 16.8%. To clarify if any components were left after the extraction procedure, extraction of disposed precipitates was performed. Hyperforin and hypericin were not detected; only rutin, hyperoside, and isoquercitrin (with maximum 8.6%) were calculated.

HPME was examined for phloroglucinols (hyperforin), naphthodianthrones (hypericin, pseudohypericin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin, quercetin, and amentoflavone), and quinic acid derivative (chlorogenic acid). The analysis was performed using HPLC-DAD ($n = 9$) every month for a period of 6 months. Validation of the analysis conditions— injection repeatability (maximum for hypericin 0.06% RSD), stability of reference standards in a day (maximum for hyperforin 1.7% RSD) and interdays (maximum for hypericin 21% RSD)—was performed. The chromatogram and RT values are given in Figs. 1 and 2. All 10 compounds were in the same exact order as in the other studies with aspect of retention time (RT) (Figs. 1 and 2) [13,17,25]. The analyzed amounts of the components are in accordance with the literature data [13,17,25]. Only the amounts of hypericins and hyperforin were observed to have increased as the studied plant material was collected from a cultivated plant population of a composition enrichment study.

Results of the analysis for 6 months are summarized in Tables 3–5, within corresponding cases.

Mainly hypericin has been indicated as the marker compound of the plant to be standardized with in pharmacopeias, monographs, and related literature [1,5]. However, recent studies have shown that all constituents might directly or indirectly contribute to bioactivity [12,16,20,21]. Considering this controversy, all constituents in the total extract were regarded within commonly encountered conditions in our study.

Storage conditions were determined as follows: room condition with daylight (case 1) and room condition without

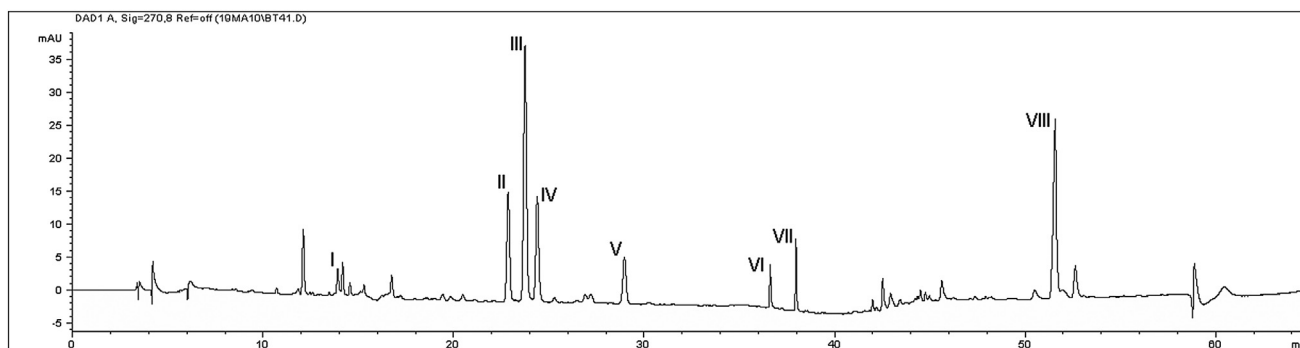


Fig. 1 – HPLC profile of MeOH extract of *Hypericum perforatum* L., monitored at 270 nm: RT = 14.11 minutes, chlorogenic acid I; RT = 22.71 minutes, rutin II; RT = 23.64 minutes, hyperoside III; RT = 24.92 minutes, isoquercitrin IV; RT = 28.79 minutes, quercitrin V; RT = 36.56 minutes, quercetin VI; RT = 37.93 minutes, amentoflavone VII; and RT = 51.63 minutes, hyperforin VIII. HPLC = high performance liquid chromatography; RT = retention time.

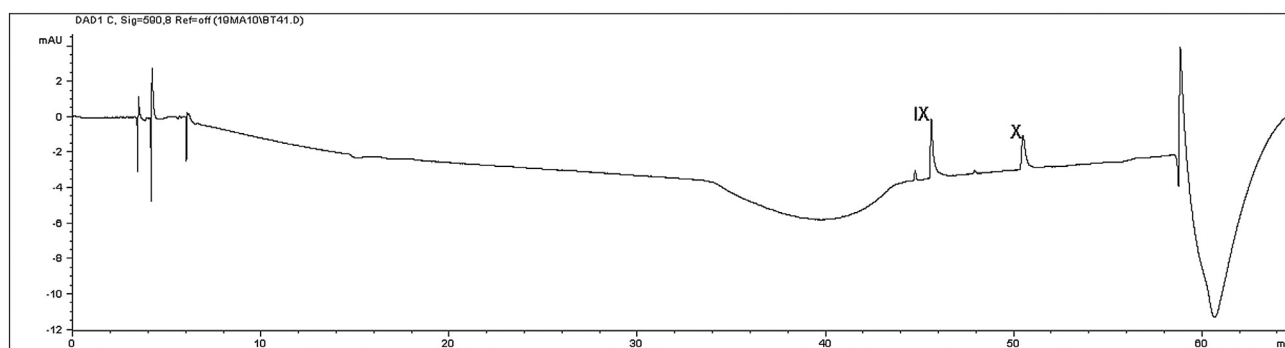


Fig. 2 – HPLC profile of MeOH extract of *Hypericum perforatum* L., monitored at 590 nm: RT = 45.87 minutes, pseudohypericin IX; and RT = 50.91 minutes, hypericin X. HPLC = high performance liquid chromatography; RT = retention time.

daylight, dark (case 2); 25°C–65% RH (case 3) and 40°C–75% RH (case 4); –20°C (case 5) and 4°C (case 6). The conditions were chosen to investigate the effects of temperature, humidity, and light. The effect of light on HPME was observed in case 1 and case 2. Cases 3 and 4 were set up to investigate the percentage of degradation, based on ICH guidelines [24]. Climate cabins were used to show the effect of heat and humidity on the percentage of degradation under extreme conditions. The potential protective effect of cold (low) temperatures on the stability of components was examined at –20°C and 4°C. The results are compiled within relevant cases.

Under light and dark room conditions, all compounds except chlorogenic acid degraded by > 24% within 4 months (results are presented in Table 3). At the end of the 4th month, keeping the extract in the dark at room condition led to a significant improvement in the stability of pseudohypericin, but not for hypericin and hyperforin. Flavonoids, except hyperoside, were highly durable for 4 months in the dark at room condition, but lost their stability at the 6th month in both dark and light conditions. The dark condition provided improved stability when compared to light for 4 months. At the 6th month, the degradative effect of time overwhelmed the protective effect of dark on the stability of flavonoids.

Under the 25°C–65% RH and 40°C–75% RH conditions, flavonoids and chlorogenic acid degraded more than hypericin and hyperforin did between the 4th month and 6th month (Table 4). Therefore, the discussion of our study mainly focused on the values of the 4th month and 6th month. At 25°C–65% RH, the degradation percentages were similar to those at room condition with or without daylight. The extreme condition of 40°C–75% RH gave the highest degradation values. Pseudohypericin, hypericin, and hyperforin were the compounds that degraded the most in 40°C–75% RH, reaching values of 65%, 85%, and 69%, respectively, at the end of the 6th month. Increase in humidity and temperature enhanced the degradation.

The breakdown of pseudohypericin and hypericin was close at the end of the 4th month for both –20°C and 4°C as presented in Table 5. By contrast, at the 6th month, differentiation in –20°C resembled that in 25°C–65% RH. At the 4th month for –20°C, 4°C, and dark room conditions, similar results were observed for pseudohypericin and hyperoside—showing that cold conditions could not provide significant protective effects for 4 months. Flavonoids (except hyperoside) were more stable at –20°C compared to 4°C for 4 months; however, at the end of the 6th month the degradation

Table 3 – Degradation percentages of constituents at room conditions of daylight and darkness during 6 months of stability trial (n = 3).

	t_0	Room condition (daylight)						Room condition (darkness)					
		1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month	1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month
		mg/g	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)
Chlorogenic acid	5.0 ± 0.14	5.1 ± 0.5 (-3.0)	5.2 ± 0.7 (-3.8)	5.2 ± 1.7 (-4.6)	5.2 ± 0.5 (-5.0)	4.0 ± 3.5 (18.6)	3.8 ± 0.6 (24.5)	5.4 ± 0.5 (-8.7)	5.8 ± 0.7 (-16.7)	6.2 ± 1.7 (-24.8)	6.7 ± 0.6 (-34.2)	5.0 ± 0.7 (-1.1)	3.6 ± 0.2 (27.5)
Rutin	20.7 ± 0.02	20.2 ± 1.6 (2.2)	19.4 ± 1.9 (6.1)	18.6 ± 4.8 (-4.6)	17.1 ± 1.2 (17.4)	16.2 ± 10.1 (21.5)	12.3 ± 1.8 (40.6)	20.7 ± 1.6 (-0.4)	20.82 ± 1.9 (-0.8)	20.9 ± 4.8 (-1.2)	21.0 ± 2.0 (-1.4)	15.2 ± 1.8 (26.3)	11.3 ± 0.2 (45.2)
Hyperoside	38.8 ± 0.06	33.0 ± 2.9 (14.9)	28.0 ± 2.7 (27.8)	26.0 ± 7.1 (10.0)	22.1 ± 1.6 (43.0)	20.3 ± 12.2 (47.5)	17.4 ± 2.5 (55.1)	34.4 ± 2.9 (11.3)	32.0 ± 2.7 (17.5)	29.6 ± 7.1 (23.7)	27.1 ± 2.5 (30.1)	19.0 ± 2.2 (51.1)	16.1 ± 0.4 (58.6)
Isoquercitrin	19.5 ± 0.32	18.0 ± 1.5 (7.7)	16.6 ± 1.8 (14.8)	15.2 ± 4.3 (33.0)	14.9 ± 1.1 (23.5)	11.0 ± 8.5 (43.5)	10.8 ± 1.5 (44.4)	19.0 ± 1.5 (2.5)	18.8 ± 1.8 (3.6)	18.6 ± 4.3 (4.6)	18.4 ± 1.7 (5.5)	13.5 ± 1.5 (30.7)	10.0 ± 0.4 (48.9)
Quercitrin	9.9 ± 0.04	9.5 ± 0.8 (3.7)	9.1 ± 1.0 (7.8)	8.9 ± 2.3 (22.0)	8.8 ± 0.6 (11.1)	8.3 ± 5.1 (16.3)	6.3 ± 1.0 (36.4)	10.2 ± 0.8 (-3.4)	10.4 ± 1.0 (-5.4)	10.6 ± 2.3 (-7.4)	10.8 ± 1.0 (-9.7)	7.8 ± 0.9 (21.1)	5.7 ± 0.2 (41.8)
Quercetin	3.2 ± 0.02	3.0 ± 0.2 (6.2)	2.8 ± 0.3 (12.5)	2.6 ± 0.9 (9.8)	2.5 ± 0.2 (23.2)	2.4 ± 1.3 (26.0)	1.8 ± 0.3 (42.7)	3.2 ± 0.2 (1.2)	3.12 ± 0.3 (2.4)	3.1 ± 0.9 (3.7)	3.0 ± 0.3 (5.0)	2.2 ± 0.3 (30.0)	1.7 ± 0.1 (47.0)
Amentoflavone	6.0 ± 0.08	5.6 ± 0.5 (6.2)	5.2 ± 0.4 (12.9)	4.8 ± 1.4 (18.7)	4.5 ± 0.4 (25.4)	3.3 ± 2.5 (45.0)	3.1 ± 0.5 (48.0)	5.9 ± 0.5 (1.8)	5.76 ± 0.4 (3.5)	5.7 ± 1.4 (5.2)	5.6 ± 0.5 (6.8)	3.9 ± 0.5 (34.6)	2.9 ± 0.1 (51.1)
Pseudohypericin	8.5 ± 0.08	6.4 ± 1.0 (24.4)	5.8 ± 0.4 (31.5)	5.4 ± 1.0 (19.6)	5.4 ± 0.8 (35.9)	5.2 ± 3.0 (38.2)	4.9 ± 1.2 (42.5)	7.8 ± 1.0 (7.8)	7.6 ± 0.4 (10.2)	7.4 ± 1.0 (12.5)	7.1 ± 0.9 (16.4)	5.2 ± 0.8 (38.6)	4.6 ± 0.3 (46.1)
Hypericin	2.9 ± 0.02	2.2 ± 0.2 (22.9)	1.8 ± 0.1 (36.9)	1.5 ± 0.3 (35.9)	1.3 ± 0.2 (53.1)	1.3 ± 0.9 (55.9)	1.2 ± 0.2 (57.1)	2.2 ± 0.2 (22.9)	1.8 ± 0.1 (36.9)	1.7 ± 0.3 (40.4)	1.6 ± 0.3 (42.9)	1.5 ± 0.4 (46.0)	1.1 ± 0.1 (62.6)
Hyperforin	69.8 ± 0.38	54.0 ± 2.3 (22.6)	44.0 ± 7.4 (36.9)	32.0 ± 4.8 (47.5)	28.0 ± 3.4 (59.8)	25.5 ± 6.9 (63.5)	21.9 ± 10.9 (68.7)	56.0 ± 2.3 (19.8)	46.0 ± 7.4 (34.1)	36.0 ± 4.8 (48.4)	25.8 ± 7.9 (63.0)	24.0 ± 12.2 (65.7)	23.5 ± 5.8 (66.4)

t_0 = starting point; Deg % = degradation percentage; ± = standard deviation.

Table 4 – Degradation percentages of constituents in 25°C–65% RH and 40°C–75% RH conditions during 6 months of stability trial (n = 3).

	t_0	25°C–65% RH						40°C–75% RH					
		1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month	1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month
		mg/g	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)
Chlorogenic acid	5.0 ± 0.14	5.1 ± 0.5 (-2.2)	5.1 ± 0.5 (-2.6)	5.1 ± 0.3 (-3.0)	5.1 ± 0.7 (-3.0)	4.8 ± 2.1 (3.5)	3.3 ± 0.3 (32.8)	4.8 ± 0.5 (3.4)	4.6 ± 1.4 (7.4)	4.4 ± 1.9 (11.5)	4.1 ± 1.0 (18.0)	2.9 ± 0.5 (41.0)	2.1 ± 0.5 (57.3)
Rutin	20.7 ± 0.02	20.0 ± 2.0 (3.2)	19.0 ± 1.7 (8.0)	18.0 ± 0.8 (12.9)	16.1 ± 2.5 (22.0)	14.8 ± 5.5 (28.5)	11.0 ± 0.5 (46.6)	20.2 ± 1.8 (2.2)	19.0 ± 6.2 (8.0)	18.2 ± 7.8 (11.9)	17.5 ± 3.9 (15.1)	11.9 ± 1.7 (42.4)	9.9 ± 2.1 (52.1)
Hyperoside	38.8 ± 0.06	32.0 ± 3.9 (17.5)	26.0 ± 2.5 (33.0)	22.0 ± 1.2 (43.3)	21.3 ± 3.2 (45.0)	19.0 ± 6.9 (51.0)	15.7 ± 1.0 (59.6)	31.0 ± 3.1 (20.1)	27.0 ± 9.0 (30.4)	25.0 ± 10.8 (35.5)	21.5 ± 4.4 (44.6)	14.3 ± 1.9 (63.0)	13.1 ± 2.7 (66.3)
Isoquercitrin	19.5 ± 0.32	18.0 ± 1.8 (7.7)	16.0 ± 1.6 (17.9)	15.0 ± 0.7 (23.0)	14.1 ± 2.2 (27.5)	13.0 ± 4.6 (33.1)	9.8 ± 0.5 (49.9)	17.4 ± 1.5 (10.7)	16.8 ± 5.6 (13.8)	16.2 ± 6.7 (16.9)	15.0 ± 3.1 (23.3)	10.4 ± 1.4 (46.5)	8.4 ± 1.7 (57.1)
Quercitrin	9.9 ± 0.04	9.5 ± 0.9 (3.9)	9.1 ± 0.8 (7.8)	8.7 ± 0.4 (11.6)	8.3 ± 1.4 (15.5)	7.4 ± 2.8 (24.6)	5.6 ± 0.3 (42.9)	9.5 ± 0.8 (3.7)	9.3 ± 3.1 (5.7)	9.2 ± 3.7 (6.7)	9.0 ± 2.0 (8.6)	5.9 ± 0.9 (39.8)	4.8 ± 1.0 (51.2)
Quercetin	3.2 ± 0.02	3.0 ± 0.3 (6.2)	2.8 ± 0.3 (12.5)	2.6 ± 0.2 (18.7)	2.5 ± 0.5 (22.1)	2.2 ± 0.8 (30.2)	1.7 ± 0.1 (46.9)	3.2 ± 0.1 (-0.1)	3.2 ± 1.3 (-0.1)	3.2 ± 1.7 (-0.1)	3.2 ± 0.9 (0.1)	2.3 ± 0.3 (29.5)	1.9 ± 0.4 (40.1)
Amentoflavone	6.0 ± 0.08	5.6 ± 0.6 (6.9)	5.2 ± 0.5 (13.6)	4.8 ± 0.3 (20.3)	4.3 ± 0.7 (27.3)	3.9 ± 1.4 (35.0)	2.9 ± 0.2 (51.3)	5.5 ± 0.6 (7.9)	5.3 ± 2.1 (11.2)	5.2 ± 2.4 (12.9)	5.1 ± 1.1 (15.2)	3.4 ± 0.5 (42.8)	2.8 ± 0.6 (52.4)
Pseudohypericin	8.5 ± 0.08	7.6 ± 1.7 (10.2)	6.8 ± 0.4 (19.6)	6.0 ± 0.2 (29.1)	5.1 ± 0.2 (39.2)	4.7 ± 1.3 (44.5)	4.6 ± 0.1 (45.3)	5.6 ± 1.0 (33.8)	5.0 ± 0.7 (40.9)	4.4 ± 1.2 (48.0)	3.8 ± 1.3 (54.7)	3.0 ± 0.5 (64.4)	2.9 ± 0.7 (65.4)
Hypericin	2.9 ± 0.02	2.5 ± 0.3 (13.8)	2.0 ± 0.2 (29.9)	1.7 ± 0.04 (40.4)	1.4 ± 0.3 (51.4)	1.3 ± 0.6 (54.1)	1.2 ± 0.1 (58.5)	1.8 ± 0.1 (36.9)	1.6 ± 0.1 (44.0)	0.9 ± 0.1 (68.5)	0.6 ± 0.01 (79.0)	0.6 ± 0.1 (80.3)	0.4 ± 0.0 (84.8)
Hyperforin	69.8 ± 0.38	62.0 ± 4.0 (11.2)	58.0 ± 1.9 (16.9)	54.0 ± 4.8 (22.6)	50.86 ± 1.0 (27.1)	43.8 ± 16.6 (37.3)	40.6 ± 4.2 (41.8)	58.0 ± 7.6 (16.9)	48.0 ± 4.2 (31.2)	34.0 ± 6.1 (51.3)	27.0 ± 4.5 (61.3)	23.2 ± 4.2 (66.8)	22.0 ± 4.0 (68.5)

t_0 = starting point; Deg % = degradation percentage; RH = relative humidity; ± = standard deviation.

Table 5 – Degradation percentages of constituents in -20°C and 4°C conditions during 6 months of stability trial ($n = 3$).

mg/g	t_0											
	-20°C						4°C					
	1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month	1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month
mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)	mg/g (Deg %)
Chlorogenic acid	5.0 ± 0.14	5.4 ± 1.1 (-8.7)	6.0 ± 0.8 (-20.7)	7.20 ± 0.8 (-45.2)	6.3 ± 0.3 (-27.5)	4.5 ± 0.9 (8.7)	5.5 ± 0.5 (-10.7)	6.0 ± 0.6 (-20.7)	6.5 ± 3.1 (-30.8)	7.2 ± 0.1 (-43.9)	6.7 ± 1.3 (-34.0)	3.8 ± 0.5 (24.0)
Rutin	20.7 ± 0.02	20.6 ± 4.4 (0.3)	20.6 ± 2.4 (0.5)	20.4 ± 2.4 (1.1)	17.6 ± 0.8 (14.9)	13.5 ± 2.4 (34.5)	20.5 ± 3.2 (0.8)	20.0 ± 2.2 (3.2)	19.5 ± 8.5 (5.6)	19.0 ± 1.5 (8.1)	18.7 ± 3.8 (9.6)	11.4 ± 0.8 (44.8)
Hyperoside	38.8 ± 0.06	34.0 ± 7.9 (12.3)	32.0 ± 3.7 (17.5)	27.7 ± 3.0 (28.7)	23.5 ± 1.3 (39.3)	19.2 ± 3.3 (50.4)	34.0 ± 5.7 (12.3)	31.2 ± 3.2 (19.5)	28.4 ± 12.9 (26.8)	25.5 ± 1.9 (34.3)	24.3 ± 5.0 (37.4)	16.4 ± 1.2 (57.8)
Isoquercitrin	19.5 ± 0.32	19.3 ± 4.1 (1.2)	19.0 ± 2.1 (2.4)	18.5 ± 2.3 (5.3)	15.7 ± 0.7 (19.3)	11.9 ± 2.1 (38.9)	18.6 ± 2.9 (4.6)	18.0 ± 1.9 (7.7)	17.4 ± 8.0 (10.7)	16.9 ± 1.3 (13.4)	16.6 ± 3.3 (14.9)	10.2 ± 0.9 (47.8)
Quercitrin	9.9 ± 0.04	10.2 ± 2.1 (-3.4)	10.4 ± 1.1 (-5.4)	10.6 ± 2.6 (-7.4)	9.2 ± 0.5 (6.5)	6.8 ± 1.1 (31.3)	9.9 ± 1.4 (-0.1)	9.9 ± 1.1 (-0.3)	9.9 ± 4.1 (-0.6)	9.9 ± 0.8 (-0.8)	9.5 ± 2.0 (3.5)	5.7 ± 0.5 (42.3)
Quercetin	3.2 ± 0.02	3.2 ± 0.8 (0.6)	3.2 ± 0.4 (1.2)	3.1 ± 0.3 (2.8)	2.6 ± 0.2 (18.4)	1.9 ± 0.4 (39.6)	3.1 ± 0.5 (2.4)	3.0 ± 0.4 (5.0)	3.0 ± 1.5 (6.8)	2.9 ± 0.2 (8.9)	2.6 ± 0.5 (18.6)	1.7 ± 0.1 (45.5)
Amentoflavone	6.0 ± 0.08	5.9 ± 1.2 (0.8)	5.9 ± 0.7 (1.5)	5.8 ± 1.6 (2.2)	4.8 ± 0.3 (18.9)	3.5 ± 0.6 (41.3)	5.8 ± 1.0 (3.2)	5.6 ± 0.6 (6.2)	5.4 ± 2.4 (9.5)	5.2 ± 0.5 (12.4)	4.9 ± 1.0 (18.2)	3.0 ± 0.2 (50.5)
Pseudohypericin	8.5 ± 0.08	8.2 ± 1.8 (2.9)	8.0 ± 0.5 (5.5)	7.5 ± 1.3 (11.7)	7.3 ± 0.2 (13.7)	6.3 ± 1.1 (25.1)	8.2 ± 3.7 (3.6)	7.9 ± 0.4 (7.1)	7.6 ± 2.1 (10.7)	7.3 ± 0.8 (13.2)	7.2 ± 1.7 (14.8)	4.7 ± 0.4 (44.4)
Hypericin	2.9 ± 0.02	2.8 ± 0.7 (3.3)	2.7 ± 0.2 (6.8)	2.5 ± 0.5 (12.4)	2.3 ± 0.2 (18.4)	2.1 ± 0.4 (25.1)	2.7 ± 1.0 (4.0)	2.6 ± 0.2 (8.2)	2.5 ± 0.7 (12.4)	2.3 ± 0.3 (18.2)	2.3 ± 0.6 (21.0)	1.6 ± 0.1 (44.4)
Hyperforin	69.8 ± 0.38	88.0 ± 12.5 (-26.1)	94.0 ± 6.8 (-34.7)	98.0 ± 17.6 (-40.4)	97.1 ± 7.5 (-39.1)	77.2 ± 14.3 (-10.7)	72.5 ± 7.6 (-3.9)	73.0 ± 5.5 (-4.6)	73.6 ± 8.3 (-5.5)	74.3 ± 27.7 (-6.9)	59.7 ± 18.0 (14.5)	37.2 ± 5.5 (46.7)

t_0 = starting point; Deg % = degradation percentage; \pm = standard deviation.

percentages were similar. Flavonoids were preserved at -20°C for 4 months but not at 4°C . Almost half of all active ingredients in the extract degraded at 4°C in 6 months. At -20°C , pseudohypericin and hypericin content degraded 25% in 6 months. These results suggest that refrigeration could not keep HPME stable for 6 months. Even more important, the degradation after 6 months at -20°C indicates the necessity for storage at -80°C , although this is considered not feasible [21]. The increased amounts of chlorogenic acid and hyperforin in the 4th month for both conditions might be attributable to the effect of analogues of the molecules to the peak area. Further techniques of analysis are required to enlighten this fact.

Previous studies demonstrated that important constituents of HP, hypericin and hyperforin, are unstable compounds. They are known to be affected by light, temperature, and humidity [12,13,16–23]. Constituents could even change during the investigation and analysis step in validation studies. Our results correlate with these findings. All components of HP degrade to varying levels. Among them, hypericin and hyperforin were the most fragile. Of the phenolic acids, chlorogenic acid was more stable than flavonoids (rutin, hyperoside, isoquercitrin, quercitrin, quercetin, and amentoflavone). The degradation profile of HP dried total extract for all major constituents has been shown under common conditions for 6 months in this study. Even storage under cold conditions could not preserve the constituents. Under some conditions, the reported ineffectiveness and adverse effects could have been due to the decay of constituents in the extract. Analysis of these constituents need to be investigated in further studies.

4. Conclusion

The instability of the individual active constituents of *H. perforatum* L. has been emphasized in some analysis studies. In our study, the fluctuation of the constituents of the total extract has been demonstrated. Among the active ingredients investigated, chlorogenic acid was generally the most stable. As predicted, decay was lowest in -20°C and highest in 40°C –75% RH for all analyzed constituents. Except for hyperforin, dark condition decreases breakdown within 4 months. No significant protective effect was provided by cold conditions on the dry extract for pseudohypericin and hyperoside for 4 months. Similar decay profiles were observed both at -20°C and 4°C for pseudohypericin, hypericin, and chlorogenic acid, whereas flavonoids were preserved at -20°C for 4 months. However, at the end of 6 months, equivalent percentages of change for all constituents in all conditions show the obvious effect of time on HPME. Previous studies on *H. perforatum* extracts mainly include investigations on selected ingredient or incomparable conditions. To the best of our knowledge, this study is the first to cover all major constituents of the dried total extract under different possible cases to identify the complexities on the total extract and demonstrate the crucial breakdown of the constituents. Instability of the compounds could vitally affect the results of ongoing analyses and could be the reason for the inconsistency in efficacy trials;

in addition, unpredictable interactions and side effects could also occur.

Conflicts of interest

The authors declare no conflict of interest.

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