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RESEARCH PAPER

Analysis and Stability of the Constituents of Artichoke and St. John's Wort Tinctures by HPLC–DAD and HPLC–MS

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ABSTRACT

In continuing our investigations on tinctures, which represent both herbal drug preparations and herbal medicinal products, 40% and 60% v/v tinctures of artichoke and St. John's wort were investigated. Artichoke is largely used in hepatic disorders, while St. John's wort is an anti-inflammatory, antidepressant, and healing agent.

Both herbal drugs contain various constituents, although the compounds responsible for the main effects have not yet been completely identified. However, caffeoylquinic acids and flavones seem to be of crucial importance for the activity of artichoke, as well as flavonoids, naphthodianthrones, and phloroglucinol derivatives for St. John's wort, and they are used as marker constituents. Thus, quantification of all these constituents was performed using high-performance liquid chromatography–diode array detection (HPLC–DAD) and HPLC–mass spectrometry (MS) analyses with rutin as external standard. In addition the stability of the constituents of these tinctures from accelerated and long-term testing was also evaluated. From the results it was evidenced that constituent content depends on the solvent used for the extraction. The stability was also shown to be very different and seems to be related to the water content of the tinctures.

Key Words: *Accelerated and long-term testing; Artichoke and St. John's wort tinctures; HPLC–DAD; HPLC–MS; Stability of the constituents*

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INTRODUCTION

In continuing our studies on the content of tinctures (1), we analyzed the constituents of artichoke and St. John's wort 40% and 60% v/v tinctures and their stability was evaluated.

Artichoke is largely employed in Europe in minor hepatobiliary dysfunction and digestive complaints such as fullness and flatulence (2,3). The 10th Edition of the French Pharmacopoeia includes a monograph of the drug (4) described as the dried ground leaves of *Cynara scolymus* L., and characteristic constituents are two flavones (luteolin-7-glucoside and luteolin-7-rutinoside) and caffeoylquinic acids, e.g., the monocaffeoyl derivative chlorogenic acid and 1,5-dicaffeoylquinic acid named cynarin (5–8). Recently, antioxidative, choleretic, anticholestatic, and hepatoprotective effects as well as inhibition of cholesterol synthesis have been demonstrated (3), and luteolin derivatives seem to be the major components responsible for the cited effects (9).

St. John's wort is largely used as an antiinflammatory and healing agent (10–12) and recently its extracts have found a wide application in Europe and the United States for their antidepressant activity (13–16). The 3rd Edition of the European Pharmacopoeia (17) includes a monograph of the drug, which consists of the dried aerial part of *Hypericum perforatum* L. collected shortly before or during the flowering period. The drug contains different constituents with documented biological and pharmacological activities such as flavonols (principally rutin and hyperoside), phloroglucinol derivatives (hyperforin, adhyperforin), and naphthodianthrones (hypericin, pseudohypericin), the latter used for standardization (18,19).

EXPERIMENTAL

Chemicals

Acetonitrile, MeOH, and absolute EtOH were HPLC grade from Merck (Darmstadt, Germany); 85% *ortho*-phosphoric acid was analytical grade 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).

Standards

Indena Research Laboratories (Settala, Milan, Italy) kindly provided rutin (international standard, batch n. K12408717, standard purity 88.17% considering the content of residual solvents, moisture, and amount of impurities). Chlorogenic acid, hypericin, hyperoside, isoquercitrin, luteolin, luteolin-7-glucoside, quercetin, and quercitrin were purchased from Extrasynthese (Genay, France).

Plant Materials

Commercial samples of herbal drugs were kindly offered by Aboca S.p.A. (Sansepolcro, Arezzo, Italy), i.e., *C. scolymus* L. (dried leaves, lot n. 55634) and *H. perforatum* L. (aerial parts, lot n. 62542).

Sample Preparation

Tinctures were obtained according to the European Pharmacopoeia (20) and stored as previously reported (1).

HPLC Analysis

Apparatus

The high-performance liquid chromatography (HPLC) system consisted of an 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 an HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA). 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–diode array detection (DAD) analysis. Mass spectrometer conditions were optimized in order to achieve maximum sensitivity electrospray ionization (ESI) values. The same column, time period, and flow rate were used. Since phosphoric acid was not suitable for HPLC–mass spectrometry (MS) operations, separation was performed using aqueous formic acid, without appreciable variations in the chromatographic profile.

Mass spectrometry operating conditions were: gas temperature 350°C at a flow rate of 10 L/min; nebulizer pressure 30 psi; quadrupole temperature 30°C; capillary voltage 3500 V. Full scan spectra from m/z 100 to 800 in the positive ion mode were

obtained (scan time 1 sec). Injected volume of samples was 25 μL solution.

Chromatography Conditions

Analyses of artichoke tinctures were carried out at 26°C on a LiChrosorb RP-18 column (5 μm, 250 mm × 4 mm i.d.; Merck, Darmstadt, Germany) equipped with a precolumn LiChrosorb RP-18 (5 μm, 10 mm × 4 mm i.d.; Merck, Darmstadt, Germany). The mobile phase was a four-step linear solvent gradient CH_3CN/H_2O (see Table 1) with H_3PO_4 (pH 3.0) during a 30-min period at a flow rate of 1.3 mL/min.

Analyses of St. John's wort tinctures were carried out at 26°C on a 214 TP 54 Protein C4 RP-18 (5 μm, 250 mm × 4 mm i.d.; Vydac, Hesperia, CA, USA). The mobile phase was a four-step linear solvent gradient $CH_3CN/MeOH/H_2O$ (see Table 2) with H_3PO_4 (pH 1.8) during a 40-min period at a flow rate of 1 mL/min.

Table 1

Time Table of Mobile Phase Used to Analyze the Artichoke Tinctures

Time (min)	% H_2O	% CH_3CN	Flow (mL/min)
0.10	88.0	12.0	1.30
10.00	82.0	18.0	1.30
15.00	82.0	18.0	1.30
30.00	55.0	45.0	1.30
35.00	0.0	100.0	1.30
42.00	0.0	100.0	1.30
50.00	88.0	12.0	1.30

Table 2

Time Table of Mobile Phase Used to Analyze St. John's Wort Tinctures

Time (min)	% H_2O	% CH_3OH	% CH_3CN	Flow (mL/min)
0.0	100.0	0.0	0.0	1.00
10.00	85.0	0.0	15.0	1.00
30.00	70.0	10.0	20.0	1.00
40.00	25.0	10.0	65.0	1.00
55.00	20.0	10.0	70.0	1.00
57.00	5.0	15.0	80.0	1.00
60.00	100.0	0.0	0.0	1.00

Injected volume of samples was 25 μL solution. Ultraviolet (UV)–vis spectra were recorded in the range 190–600 nm, and chromatograms were acquired at 254, 270, 350, and 590 nm. Peaks were detected at 350 nm for artichoke tinctures and at 270 nm for St. John's wort tinctures. Tinctures were filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane before HPLC analysis.

A typical artichoke chromatogram (60% v/v) is reported in Fig. 1. The t_R values for chlorogenic acid (**1a**), luteolin-7-rutinoside (**2a**), luteolin-7-glucoside (**3a**), isochlorogenic acid (**4a**), cynarin (**5a**) were 6.2, 16.4, 18.2, 20.6, 22.1 min, respectively.

A typical St. John's wort chromatogram (60% v/v) is reported in Fig. 2. The t_R values for rutin (**1s**), hyperoside (**2s**), isoquercitrin (**3s**), quercitrin (**4s**), quercetin (**5s**), hypericin (**6s**), pseudohypericin (**7s**) were 15.0, 15.2, 16.2, 16.7, 20.6, 42.8, 43.7 min, respectively.

Identification of Constituents and Peaks Purity

Identification of all constituents was performed by HPLC–MS analysis and/or by comparing the retention time of the peaks in the extracts with those of the authentic reference samples. The purity of peaks was checked with a diode array detector coupled to the HPLC system by comparing the UV spectra of each peak to those of authentic reference samples and/or by examination of the MS spectra.

Linearity

The linearity range of responses was determined at five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from 0.10 to 2.5 μg ($r > 0.99$).

Repeatability

To evaluate the repeatability, six samples of each tincture from the same batch were analyzed by HPLC. The contents of each constituent were evaluated to calculate the relative standard deviation (chlorogenic acid 1.9%, cynarin 2.1%, hypericin 1.8%, hyperoside 3.7%, isoquercitrin 1.5%, luteolin-7-glucoside 2.4%, luteolin-7-rutinoside 2.6%, quercetin 1.4%, quercitrin 1.5%, rutin 3.0%).

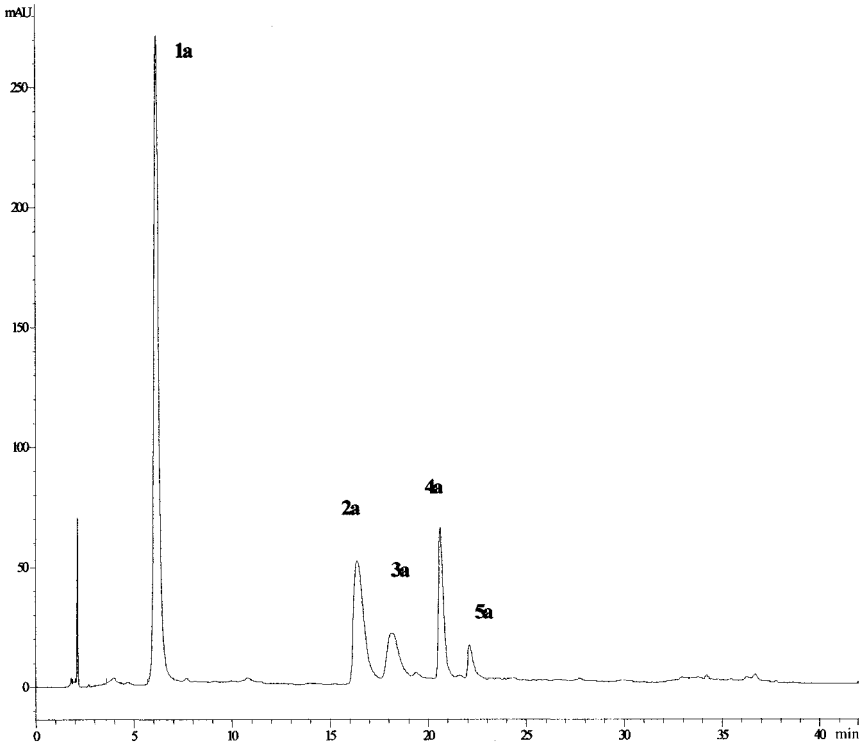

Figure 1. Chromatogram of 60% v/v artichoke tincture.

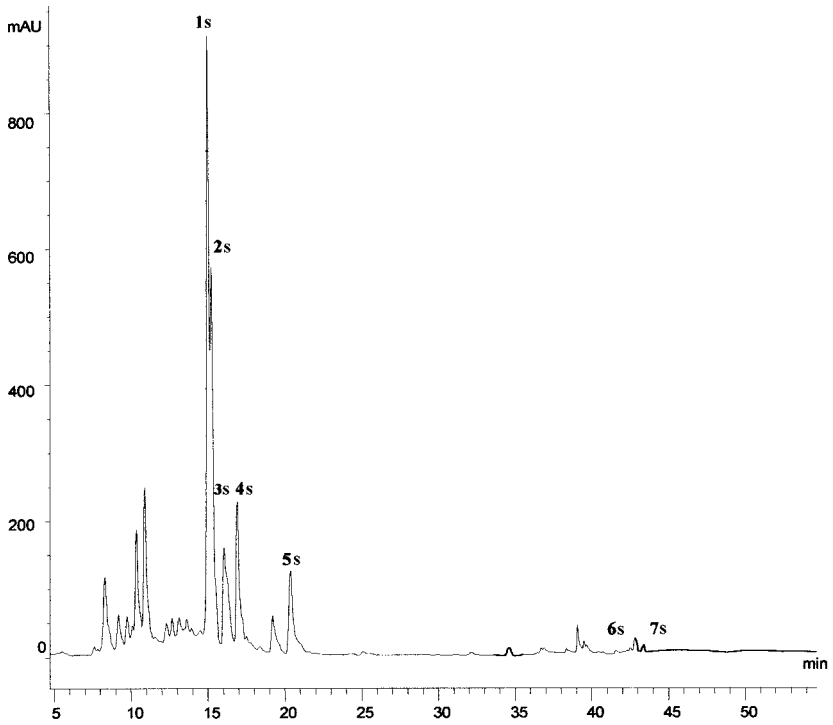

Figure 2. Chromatogram of 60% v/v St. John's wort tincture.

Reproducibility

To evaluate the reproducibility of the injection integration, the standard solutions of rutin (2.5 μg/25 μL) and of each tincture sample were injected six times. The relative standard deviation values were calculated as chlorogenic acid 1.4%, cynarin 1.1%, hypericin 1.2%, hyperoside 1.4%, isoquercitrin 1.3%, luteolin-7-glucoside 1.0%, luteolin-7-rutinoside 0.85%, quercetin 2.0%, quercitrin 1.9%, rutin 1.0%.

Quantitation of Constituents

All the tinctures were analyzed in triplicate and a calibration graph with six datapoints of external standard was used. The contents of constituents were calculated taking into account the mean of the response factor of rutin in the reference solutions, i.e., $\text{area/concentration}_{(\text{mg/mL})} \times \text{purity}/100$, and the response factor of the considered constituent relative to rutin (RRF). This value was determined by calculating the ratio between the average response factor of each compound and the average response factor of rutin at 350 or 270 nm. Monocaffeoylquinic acid derivatives were evaluated as chlorogenic acid, dicaffeoylquinic acid derivatives as cynarin, luteolin-7-rutinoside as luteolin-7-glucoside, and pseudohypericin as hypericin.

Evaluation of the Results Obtained from Tinctures Subjected to Accelerated Storage

Data obtained from the accelerated testing (40°C, 50°C, 60°C±2°C) were used to predict the shelf-life at 25°C, by the Arrhenius law (21), of only a few constituents. Mean concentration–time data for each compound, obtained from the accelerated storage, were analyzed by linear and exponential regression analysis and the kinetic order was determined only if the slope of the regression line was significant ($r > 0.900$).

RESULTS AND DISCUSSION

As a first step in our investigation, the HPLC analysis of the 40% v/v and 60% v/v tinctures were performed to evaluate the content of the constituents and thus the influence of alcoholic degree on the extractability and stability of the tested constituents. Tincture samples were filtered through a cartridge-type sample filtration unit with a PTFE membrane before the direct HPLC analysis. Simple reverse HPLC methods that optimized the separation of all constituents were carried out (Figs. 1 and 2) and rutin was used as external standard. Good linearity of the calibration curves was achieved between 0.1 and 2.5 μg ($r > 0.99$); the repeatability and reproducibility of the method were satisfactory. We fixed UV detection at 350 nm for artichoke and 270 nm for St. John's wort, as all the constituents of the preparations showed appreciable absorbance at these wavelengths. Peaks were identified by comparing their Rt value with those of authentic samples, and by comparing their UV and MS data with those reported in the literature (see Tables 3 and 4). Both the 40% and 60% v/v tinctures of each herbal drug showed thin-layer chromatography (TLC) profiles and chromatograms qualitatively similar. However, the quantitative contents were quite different, as shown in Tables 5 and 6.

Both artichoke tinctures showed the presence of two flavone glycosides and three quinic acid derivatives. However, during storage, two additional

Table 3

Retention Times (t_R), UV, and MS Data of the Constituents of Artichoke Tinctures

No.	Constituent	t_R (min)	UV Data (nm)	MS Data (m/z)	Source Ref.
1a	Chlorogenic acid	6.2	236, 327	355, 337	24
2a	Luteolin-7-rut	16.4	255, 348	585, 607, 449, 287	25
3a	Luteolin-7-glc	18.2	255, 348	449, 671, 287	25
4a	Isochlorogenic acid	20.6	236, 327	517, 501	24
5a	Cynarin	22.1	236, 327	517, 501	Authentic sample
	Chlorogenic acid isomer	6.0	236, 327	355, 337	24
	Luteolin	26.7	255, 348	287	25

Table 4

Retention Times (t_R), UV, and MS Data of the Constituents of St. John's Wort Tinctures

No.	Constituent	t_R (min)	UV Data (nm)	MS Data (m/z)	Source Ref.
1s	Rutin	15.0	257, 298 (sh), 358	633, 611, 465, 303	Authentic sample
2s	Hyperoside	15.2	257, 299 (sh), 362	303, 465, 487, 503	Authentic sample
3s	Isoquercitrin	16.2	257, 267 (sh), 295 (sh), 358	465, 487, 303	Authentic sample
4s	Quercitrin	16.7	256, 265 (sh), 301 (sh), 350	303, 449, 471	Authentic sample
5s	Quercetin	20.6	270, 310 (sh), 371	303	Authentic sample
6s	Hypericin	42.8	270, 590	503	Authentic sample
7s	Pseudohypericin	43.7	270, 590	519	26

peaks were evidenced. The first one (Rt = 6.0) was represented by an isomer of chlorogenic acid, probably formed by the hydrolysis of dicaffeoylquinic derivatives. In addition, a peak (Rt = 26.7) corresponding to the aglycone luteolin was also found, due to the hydrolysis of the saccharide moieties of the luteolin glycosides.

In the chromatograms of St. John's wort tinctures, peaks corresponding to flavonols, naphthodianthrones, and quinic acid derivatives were evidenced. No peaks of phloroglucinols were found, probably due to the polarity of the solvents used in the extraction or to their unstability in aqueous solvents (22). In addition, quinic acid derivatives of St. John's wort tinctures were not considered in this investigation because they do not seem to be involved in the antidepressant activity.

All the tinctures were subjected to long-term testing at 25°C±2°C for six months, and also to accelerated testing at three different temperatures (40°C, 50°C, and 60°C±2°C) for three months. Stability was evaluated by the analysis of the marker constituents, i.e., flavones and quinic acid derivatives of artichoke and flavonols and naphthodianthrones considered as total hypericins of St. John's wort.

The analyses of tinctures subjected to long-term testing were carried out in triplicate every 30 days. Limits of acceptance for the stability evaluation were considered 90%, i.e., 10% potency loss from the initial value of the sample for flavonoids considered as markers. The limit for known active constituents was considered 95%, i.e., 5% potency loss from the initial assay value of the sample as suggested by the literature (23).

In these studies the total content of marker constituents was also considered because the biological effects of herbal drugs and their preparations are generally considered to arise from the whole phytocomplex rather than a single constituent (Figs. 3 and 4).

Both artichoke tinctures showed a similar total content of quinic acid derivatives, however, during storage their content gave rise to different decomposition percentages in the two tinctures, as reported in Table 5. The flavone content was quite different: in the 60% v/v tinctures luteolin derivatives represent about 20 mg/100 mL tincture, while in 40% v/v tincture the content was one-half.

St. John's wort 40% and 60% v/v tinctures were less stable than artichoke tinctures (see Table 6). These tinctures showed a very different content of flavonols and hypericins and, as expected, they gave rise to different decomposition percentages (see Table 6 and Fig. 4).

The results obtained by the accelerated degradation methods were analyzed by the Arrhenius law to predict the shelf-life of single constituents at 25°C. Mean concentration–time data for metabolite content were analyzed by linear and exponential regression analysis and decomposition was assumed if the slope of the regression line was significant. Only a few constituents showed a degradation rate fitting with a first-order kinetic, and the data obtained after storage at 25°C±2°C confirmed a very short shelf-life (see Table 7). These results were in approximate agreement with the shelf-life found by long-term testing. Thus, the data obtained from accelerated degradation methods examined a decomposition of the phytocomplex, but the rate of degradation did not fit the law. Therefore, only long-term testing was used to establish the t_{90} shelf-life of each tincture.

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Table 5

Content and Residual Percentage of Artichoke Tinctures (60% and 40% v/v) After Thermal Stability Testing (25°C)

Storage Period (days)	Chlorogenic Acid		Chlorogenic Acid Isomer		Luteolin-7-rut		Luteolin-7-glc		Isochlorogenic Acid		Cynarin		Luteolin	
	mg/100 mL± SD	%	mg/100 mL± SD	%	mg/100 mL± SD	%	mg/100 mL± SD	%	mg/100 mL± SD	%	mg/100 mL± SD	%	mg/100 mL± SD	%
Artichoke Tincture, 60% v/v														
0	30.65±1.85		—		14.38±1.65		5.31±0.98		7.19±1.01		2.16±0.75		—	
60	29.73±1.05	97.0	—		13.52±2.07	94.0	5.22±1.46	98.3	7.10±2.01	98.7	2.09±1.01	97	—	
90	29.42±1.92	96.0	1.03±1.12	100	12.50±1.78	86.9	5.17±1.86	97.4	6.85±1.75	95.3	1.85±1.35	86	1.43±1.36	
120	29.24±2.31	95.4	1.33±2.05	129	11.85±2.36	82.4	4.88±2.34	92.0	6.64±2.45	92.3	Not detectable		1.95±1.85	136
150	27.89±1.87	91.0	2.56±2.18	249	11.22±2.76	78.0	4.73±2.56	89.0	6.28±2.40	87.4	Not detectable		2.34±2.33	164
Artichoke Tincture, 40% v/v														
0	27.90±1.78		—		5.26±1.53		4.48±1.13		11.64±1.15		2.01±1.36		—	
60	26.62±2.35	95.4	0.35±1.05		4.97±2.03	88.4	4.42±2.07	98.6	11.19±1.70	96.1	1.92±2.01	95.4	0.25±0.95	
90	25.39±1.96	91.0	1.44±2.75	411	4.43±2.38	84.3	4.31±2.38	96.3	10.87±2.31	93.4	1.75±1.78	87.0	0.70±1.03	280
120	24.41±2.76	87.5	1.75±2.39	500	4.26±2.79	81.0	4.24±2.85	94.7	10.32±2.24	88.7	Not detectable		0.98±1.53	392

Table 6

Content and Residual Percentage of St. John's Wort Tinctures (60% and 40% v/v) After Thermal Stability Testing (25°C)

Storage Period (days)	Quercetin-3-rut		Quercetin-3-gal		Quercetin-3-glc		Quercetin-3-rha		Quercetin		Hypericins	
	mg/100 mL±SD	%	mg/100 mL±SD	%	mg/100 mL±SD	%	mg/100 mL±SD	%	mg/100 mL±SD	%	mg/100 mL±SD	%
Hypericum perforatum Tincture, 60% v/v												
0	32.63±1.51		14.05±1.82		10.21±0.93		8.51±1.12		7.95±0.95		0.15±0.12	
30	32.01±1.85	98.1	13.67±2.02	97.3	10.15±1.37	99.4	8.40±1.75	98.7	7.93±2.31	99.8	0.14±0.15	95.3
60	30.54±1.63	93.6	13.61±2.32	96.9	9.89±2.01	96.9	8.17±2.07	96.0	8.06±2.75	101.4	0.13±0.85	90.0
90	29.53±2.01	90.5	13.32±1.87	94.8	9.76±1.75	95.6	8.02±1.97	94.3	7.49±1.95	94.2	0.12±0.91	82.0
120	28.38±2.32	88.7	12.93±1.96	92.9	9.98±2.01	97.8	7.83±2.51	92.0	7.30±2.34	91.8	Not detectable	
Hypericum perforatum Tincture, 40% v/v												
0	6.03±0.95		3.15±1.85		2.17±0.98		0.85±0.60		8.51±1.34		0.09±1.07	
30	5.75±1.35	95.4	3.07±2.25	97.5	2.13±1.40	98.0	0.82±1.10	96.8	8.42±1.30	99.0	0.08±1.38	90
60	5.55±1.97	92.0	3.02±1.76	95.9	2.05±2.03	94.9	0.79±1.40	92.8	8.17±1.76	96.0	Not detectable	
90	5.22±2.01	86.5	2.98±2.35	94.7	2.03±2.75	93.4	0.77±1.75	91.3	8.01±2.01	94.1	Not detectable	
120	4.90±2.75	81.3	2.90±2.64	92.0	1.94±2.47	89.4	0.74±2.01	87.0	7.76±1.75	91.2	Not detectable	

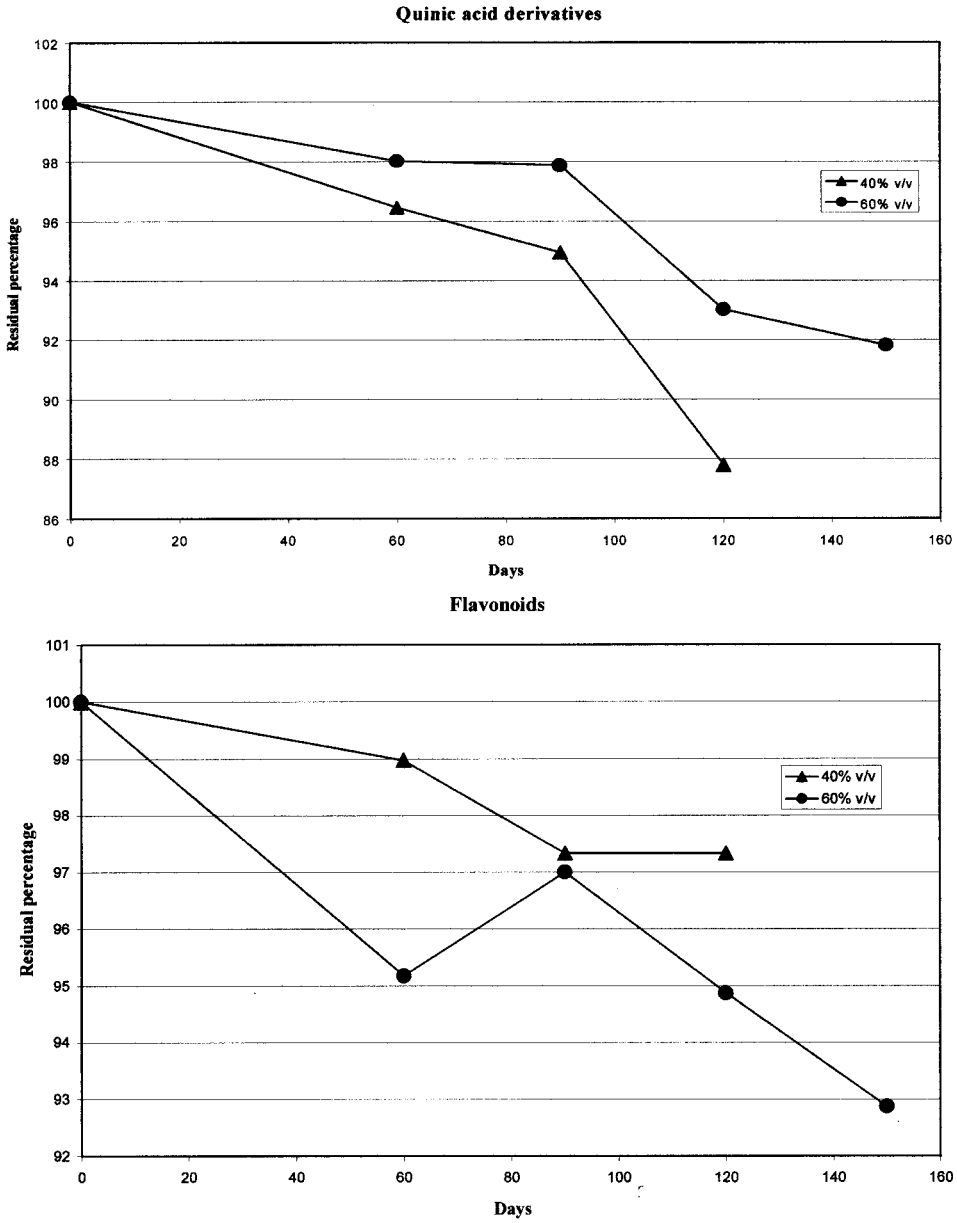

Figure 3. Residual percentage content of quinic acid derivatives and total flavonoids in artichoke tinctures (40% and 60% v/v).

CONCLUSIONS

Tinctures are generally considered one of the best liquid forms of plant extracts due to good stability of the constituents and low microbiological contamination. In the present study, tinctures of artichoke and St. John's wort, prepared according to the European Pharmacopoeia with commercial standardized herbal drugs, were investigated for the content and stability of the marker constituents. Two different alcoholic degrees, i.e., 40% and 60% v/v, were used to prepare the tinctures and to evidence differences in the quali-quantitative profile of the extractives and in maintaining phytocomplex integrity. The analysis was performed on tinctures as such, after filtration by RP–HPLC assay. The developed

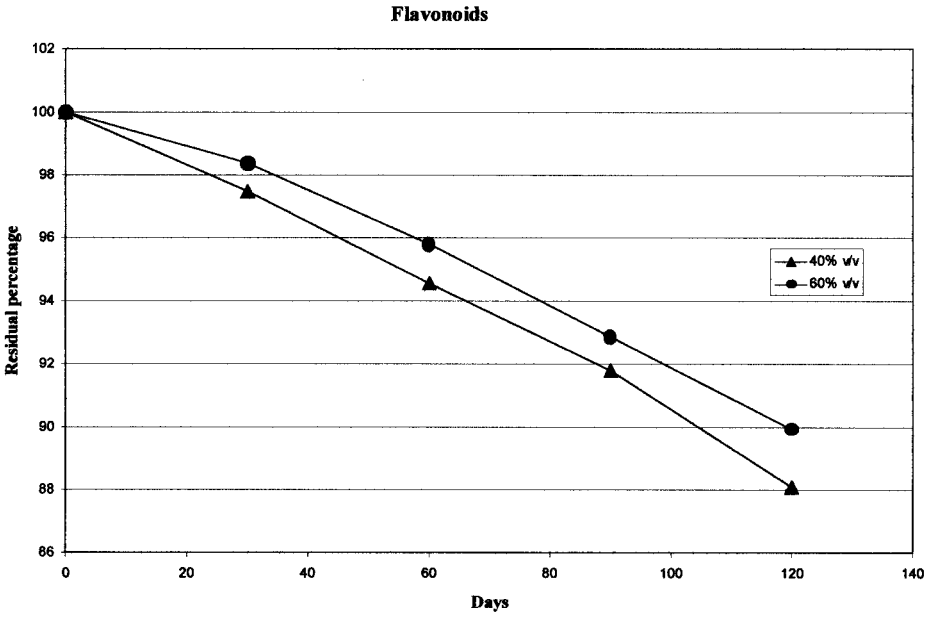

Figure 4. Residual percentage content of total flavonoids in St. John's wort tinctures (40% and 60% v/v).

Table 7

Expired and Experimental Shelf-Life of Tinctures

Tincture (Alcohol Content, %)	Constituent	Expired Shelf-Life[a] (days)	Experimental Shelf-Life[a] (days)
Artichoke (40% v/v)	Isochlorogenic acid (**4a**)	99	105
Artichoke (60% v/v)	Isochlorogenic acid (**4a**)	130	135
Artichoke (40% v/v)	Luteolin-7-rutinoside (**2a**)	64	48
Artichoke (60% v/v)	Luteolin-7-rutinoside (**2a**)	82	75
St. John's wort (40% v/v)	Rutin (**1s**)	48	45
St. John's wort (60% v/v)	Rutin (**1s**)	85	90

[a]A limit of a 10% loss from the initial assay value of the batches as suggested for the herbal drug preparations containing constituents without known therapeutic activity.

HPLC methods provided acceptable accuracy, specificity, reproducibility, and speed, together with good separation of all marker constituents, i.e., quinic acid derivatives and flavones in artichoke and flavonols and hypericins in St. John's wort.

The tinctures have been represented as some of the most versatile and widely employed herbal extracts so far, but from the above results, it is evident that they cannot be considered as extensively acceptable herbal drug preparations or herbal medicinal products because, at least for the investigated samples, the phytocomplex can be extracted only in part and the stability at room temperature is very low.

In addition, the tinctures submitted to accelerated degradation methods exhibited a decomposition of the phytocomplex, but in general the rate of degradation did not obey the Arrhenius law, therefore only long-term experiments can establish the t_{90} shelf-life.

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