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Determination of total flavonoids and quercetin in *Hyperici herba* and its aqueous, aqueous-ethanolic and oil extracts

SVETLANA KULEVANOVA[1]*
MARINA STEFOVA[2]
TRAJČE STAFILOV[2]

[1] *Institute of Pharmacognosy*
Faculty of Pharmacy, Vodnjanska 17
91000 Skopje, Republic of Macedonia

[2] *Institute of Chemistry*
Faculty of Science, POB 162,
91000 Skopje, Republic of Macedonia

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Flavonoids of *Hyperici herba* (*Hypericum perforatum* L., *Hypericaceae*) collected in southwest Macedonia, were examined. The total flavonoid content determined spectrometrically (with $AlCl_3$) was found to be 1.9%. The total flavonoid and total aglycone content varied in aqueous, aqueous-ethanolic and ethanolic extracts prepared from the same plant material. The highest value of total flavonoids (1.32 mg mL^{-1}) was found in extracts obtained with 70% ethanol, whereas for total aglycones 50% ethanol was found to be the most effective extraction solvent giving 0.84 mg mL^{-1} aglycones. The content of total and aglycone quercetin was determined using HPLC. Its content in the plant material was found to be 1.3%. Analogously to flavanoids the highest value for total quercetin (0.68 mg mL^{-1}) was also found in extracts prepared with 70% ethanol, and for aglycone 50% ethanol was most favorable and yielded 0.24 mg mL^{-1} quercetin. In olive oil extracts of *Hyperici herba*, highest yield of total flavonoids was obtained after three hours of digestion at 100 °C. Total flavonoids content in these oil extracts was 13.0 mg per 100 g.

Keywords: *Hypericum perforatum* L. (*Hypericaceae*), extracts, flavonoids, quercetin, spectrometry, HPLC

St. John's wort (*Hypericum perforatum* L., *Hypericaceae*) is used externally for treatment of various skin injuries (wounds, cuts, burns), pain in muscles, usually in the form of oil extract known as *Hyperici oleum* (1). Aqueous and aqueous-ethanolic extracts of *Hyperici herba* are used internally as sedatives and antidepressives for psychogenic disturbances such as depression, insomnia, anxiosity, *etc.* (2). All the above types of extracts are used for regulation of stomach function and for soothing stomach-ache.

The effects of *Hypericum perforatum* are due to the activity of its compounds, such as hypericin, hyperphorines, flavonoids, tannins, proantocyanidines, coumarines, various amino acids, organic acids, sterols, vitamins, *etc.* (3, 4). *Hypericum* flavonoids are derivatives of quercetin, apigenin and luteolin, present in the herb as free aglycones, dimmers (biapigenin, amentoflavon) or heterosydes (rutin, hyperin, quercitrin, isoquercitrin, *etc.*) (3, 4). Some authors emphasise that the efficiency of the extraction of flavonoids from

* Correspondence

the herb depends on the type of extraction and the solvent used (5–8), but it depends on the humidity of the plant material as well, especially when preparing oil extracts.

The flavonoids of *Hypericum preforatum* from Macedonia have not been thoroughly examined so far. The aim of this work is to analyse the content of total flavonoids and quercetin in aqueous, aqueous-ethanolic and oil extracts of the drug *Hyperici herba* from Macedonia.

EXPERIMENTAL

Materials

Plant material. – Aerial parts of the plant *Hypericum perforatum* L. (*Hypericaceae*) 15 cm long under the flowers were collected in blossom, in July 1996, on the Baba Mountain, south-west Macedonia. The material was air-dried, packed in paper bags and kept in dry and dark environment at room temperature. The sample was identified by Professor V. Matevski from the Botany Department, Faculty of Science (Skopje, Macedonia).

Chemicals. – Quercetin dihydrate and methanol (HPLC grade) were purchased from Merck (Germany). Rutin, kaempferol, myricetin and luteolin were purchased from Extrasinthese (France). All other chemicals were of *pro analysi* quality, purchased from Alkaloid (Macedonia). Aminopropyl cartridges (500 mg, Bond Elute LRC NH_2) were from Varian (USA). *Oleum hyperici* samples were the products of Alkaloid, Galafarm, Fitofarm and Galenska laboratorija (Macedonia). Olive oils were purchased from the local market.

Standard solution of quercetin (StQ, 0.5 mg g^{-1}) was prepared by dissolving 55.95 mg of quercetin in 2 mL ethyl acetate and by adding olive oil of up to 100 g. Standard solution of quercetin for HPLC analysis was prepared by dissolving an appropriate quantity of quercetin in methanol.

Apparatus. – Perkin Elmer UV-Vis spectrometer Lambda 16 (Perkin-Elmer, Germany) for spectrometric measurements and Varian HPLC system equipped with a ternary pump Model 9012 and diode-array UV-detector Model 9065 for liquid chromatography measurements were used.

Extraction procedures

Aqueous, ethanolic and aqueous-ethanolic extracts. – The extracts were prepared using dry and milled plant material (moisture content 8.3%) continuously mixed with the solvent in ratio 1:10 (*m/V*), at room temperature, over two hours. Water, water-ethanol mixtures and ethanol were used as extraction solvents and the extracts were marked as A00 (water extract), A15, A30, A50 and A70 (extracts prepared with 15, 30, 50 and 70% ethanol, respectively), and A96 (extract prepared with conc. ethanol).

Oil extracts. – They were prepared by digestion of the dried and milled material with olive oil in ratio 1:10 (*m/m*), at 100 °C, over different periods of time. The extracts were marked as B-1 to B-9 for the ones prepared during 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4 and 5 hours of digestion. Two oil extracts marked as B-15 and B-40, were prepared by exposing the samples placed in olive oil in glass vessels, to sunlight for 15 and 40 days.

For the sake of comparison four commercial samples of *Oleum hyperici* (C-1 to C-4) were included in the investigation, as well.

Analytical procedures

Total flavonoids in the drug. – Official method for spectrometric determination of flavonoids was used (9). The procedure includes hydrolysis of glycosides, extraction of the total flavonoid aglycones content with ethyl acetate and developing colour with $AlCl_3$. The absorbance is measured at 425 nm, against the solution without $AlCl_3$ being a blank.

Calibration solutions. – Calibration solutions in the concentration range of 1.0 to 10.0 μg mL^{-1} were prepared from stock solution of quercetin in methanol (100 μg mL^{-1}).

Total flavonoids in the extracts. – After hydrolysis: The extract was treated in the same manner as the drug (hydrolysis of glycosides with HCl in acetone media). The acetone hydrolysate was analysed. No hydrolysis. The extract was mixed with water in a separatory funnel and extracted with ethyl acetate. Ethyl acetate fractions were collected and dried with anhydrous Na_2SO_4, filtered and made up with ethyl acetate. Final solution was used for complex formation with $AlCl_3$ or for determination of quercetin by HPLC.

Liquid-liquid extraction of oil extracts, with hydrolysis. – Oil extract (or quercetin standard) was mixed with hexamethylenetetraamine, acetone and HCl (25%) in an Erlenmeyer flask equipped with reflux and heated in water bath for 30 min. After cooling, the mixture was mixed with water and extracted with ethyl acetate. The latter solution was used for developing the colour with $AlCl_3$ and measuring the absorbance.

Solid-phase extraction (SPE) of oil extracts. – The aminopropyl SPE cartridges were conditioned according to the procedure given in literature (7). Oil extract dissolved in heptane was deposited on the preconditioned aminopropyl column and washed with heptane. Elution was performed with 5% formic acid in acetone/methanol (1:1). After reaction with $AlCl_3$ the absorbance of the eluate was measure.

The accuracy of the liquid-liquid and SPE extracts was checked by determination of the recovery using quercetin standard solution StQ.

High performance liquid chromatography

Identification of flavanoids and determination of quercetin. – For this purpose HPLC on RP C18 column (250 x 4.6 mm, particle diameter 5 μm) was used. Twenty microliters of the samples were injected. The mobile phase consisted of water (solvent A) and methanol (B) and the flow rate was 0.7 mL min^{-1}. The elution program was as follows: 0–15 min 60% B isocratic, 15–25 min linear gradient to 70% B and isocratic 70% B to 30 min. The elution was monitored at 254 and 360 nm and the obtained data were compared with authentic samples of the respective flavonoids.

Quercetin in the drug. – Quercetin was assayed in ethyl acetate hydrolysates obtained as described above which were evaporated under low pressure, dissolved in methanol and filtered (0.45 μm, Sartorius, Germany) prior to HPLC analysis.

Quercetin was determined in aqueous, ethanolic and aqueous-ethanolic *Hypericum* extracts. Portions of the solutions obtained by procedure for determination of total flavonoids in extract were evaporated under low pressure and the residues dissolved in

methanol, filtered (0.45 μm) and subjected to HPLC analysis. For calibration purposes solutions of 0.2–1.0 mg mL^{-1} quercetin were used, prepared from the the stock solution (10 mg mL^{-1}).

RESULTS AND DISCUSSION

Identification of flavonoids

Quercetin was identified in the methanolic extract of the drug, in the ethyl acetate hydrolysates, as well as in aqueous-ethanolic extracts A15, A70 and A96. Except in ethyl acetate hydrolysates, rutin was identified in the extracts as well. The chromatogram in Fig. 1a shows the separation of a model mixture of flavonoids containing: rutin, myricetin, quercetin, luteolin and kaempferol. In the aqueous-ethanolic extract of *Hypericum perforatum* rutin (t_R = 5.31 min) and quercetin (t_R = 11.00 min) were identified (Fig. 1b), whereas in the hydrolysed ethyl acetate extract, only quercetin was found (Fig. 1c).

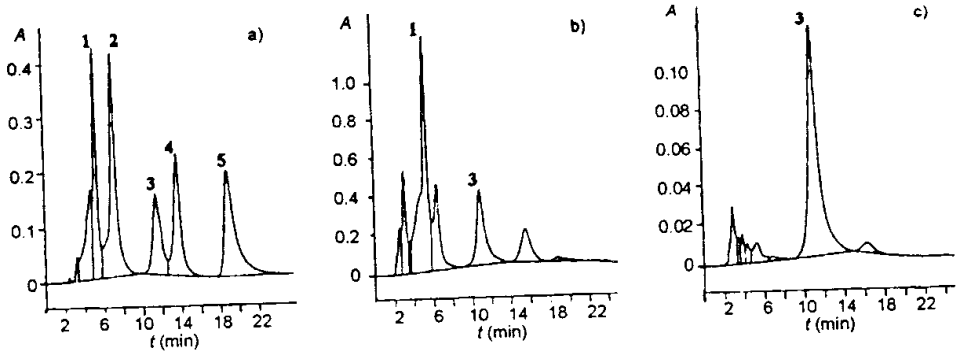

Fig. 1. HPLC chromatograms of: a) mixture of authentic samples of 1-rutin, 2-myricetin, 3-quercetin, 4-luteolin, 5-kaempferol; b) aqueous-ethanol extract of *Hyperici herba*, c) ethyl acetate extract after hydrolysis.

Spectrometric analysis of total flavonoids

In the concentration range of 1.0–10.0 μg mL^{-1} quercetin obeys Lambert-Beer's law with the following regression equation: $A = -0.016 + 0.0586\ \gamma$ (r = 0.999). The average content of total flavonoids in *Hyperici herba* expressed as quercetin was found to be 1.9% (RSD = 8.1%, n = 10). Results of total flavonoids analysis in aqueous, aqueous-ethanolic and ethanolic extracts as well as the yield of total flavonoids are given in Table I.

It can be seen that the extraction of total flavonoids varies depending on the extraction solvent, when other conditions of the extraction are maintained constant. The extraction with concentrated ethanol give results similar to those obtained with 15%- and 30%- ethanol. The yield of the extraction (quantity of total flavonoids in the extract compared to that found in the drug) ranged from 41.2 to 44.3%. More efficient extraction

Table I. Total flavonoids in the extracts determined by spectrometric method

Extract	Total flavonoid, before hydrolysis (mg mL^{-1})[a]	Yield (%)[b]	Total flavonoid, after hydrolysis (mg mL^{-1})[a]	Yield (%)[b]
A00	0.03 ± 0.00	1.3	0.15 ± 0.01	7.5
A15	0.70 ± 0.06	36.4	0.79 ± 0.06	41.2
A30	0.74 ± 0.06	38.3	0.85 ± 0.07	44.3
A50	0.84 ± 0.07	43.5	1.25 ± 0.10	65.0
A70	0.53 ± 0.04	27.7	1.32 ± 0.11	68.7
A96	0.19 ± 0.02	9.8	0.85 ± 0.07	44.0

[a] Mean ± SD (n = 5). [b] The portion of flavonoids that passed into the extract.

(65.0–68.7% of total flavonoids) was obtained when aqueous-ethanolic mixtures with ethanol content of 50 and 70% were used.

Free flavone aglycones and heterosydes have different solubility in ethanol and water and their quantity in the extracts varies considerably, which implies the need for determination of free flavone aglycones. The results given in Table I show that the increasing content of ethanol up to 70% in the extraction solvent increased and afterwork decreased the yield of flavone aglycones.

The ratio between total flavonoids and flavone aglycones content is shown in Fig. 2. It can be seen that the flavonoid pattern of the aqueous-ethanolic *Hypericum* extracts varies considerably with the ethanol content in the extraction solvent. In the extracts A15 and A30, almost the whole quantity of total flavonoids is for aglycones, whereas in A96, similar total flavonoids content has been found, but most of them in the form of heterosydes.

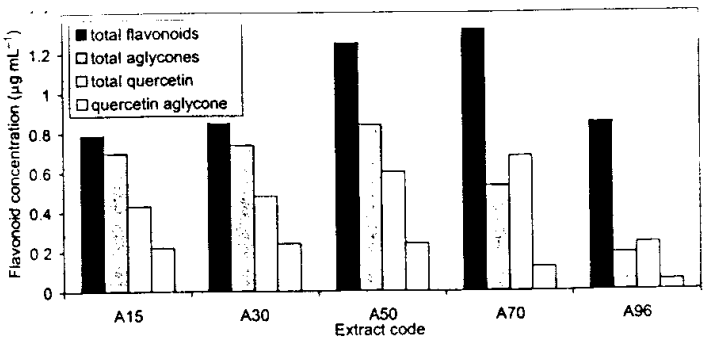

Fig. 2. Comparison of total flavonoids and total flavone aglycones in the extracts of *Hyperici herba* determined spectrometrically and total quercetin before and after hydrolysis in the extracts analysed by HPLC.

HPLC analysis of quercetin

The calibration line for quercetin was established in the concentration range of 0.2–1.0 mg mL^{-1} with the regression equation: $A = 1.1019 \cdot 10^7 \cdot \gamma$ (r = 0.9985).

The content of quercetin in *Hyperici herba* determined by HPLC is 1.3% (RSD = 8.9%, n = 5). This value counts for the total content in the drug, which is a sum of free aglycone quercetin and quercetin released from heterosydes.

The results of total quercetin determination in the aqueous-ethanolic extracts, after hydrolysis, obtained by HPLC are presented in Table II. The concentration of total quercetin ranged from 0.24–0.68 mg mL^{-1}. It is obvious that the quantity of total quercetin in the extracts increases when increasing ethanol content of up to 70% (highest concentration determined), then decreases and lowest concentration of total quercetin is found in the extract obtained with 96% ethanol as extraction solvent.

The content of free quercetin in aqueous, aqueous-ethanolic and ethanolic extracts was determined by HPLC, as well. From Table II it is evident that the content of free quercetin ranged from 0.05 mg mL^{-1} in the extract prepared with 96% ethanol, to the highest value of 0.24 mg mL^{-1} obtained with 50% ethanol.

Table II. Total quercetin in the extracts determined by HPLC method

Extract	Quercetin, before hydrolysis (mg mL^{-1})[a]	Yield (%)[b]	Quercetin, after hydrolysis (mg mL^{-1})[a]	Yield (%)[b]
A15	0.22 ± 0.02	16.8	0.43 ± 0.03	32.2
A30	0.24 ± 0.02	17.9	0.48 ± 0.04	36.5
A50	0.24 ± 0.02	18.5	0.60 ± 0.05	45.5
A70	0.12 ± 0.01	9.4	0.68 ± 0.05	51.2
A96	0.51 ± 0.04	3.9	0.24 ± 0.02	18.2

[a] Mean ± SD (n = 5). [b] The portion of flavonoids that passes into the extract.

The ratio between total quercetin and free aglycone quercetin is shown in Fig. 2. The content of total quercetin increases with 15%-, 30%-, 50%- ethanol, and highest value has been found with 70%. As for free quercetin, the results for A15, A30 and A50 are slightly increasing with remarkable decrease for extracts prepared with 70% and 96% ethanol. This implies that higher concentration of total quercetin is due to its heterosydes.

Results from the analysis of total and aglycone flavonoids determined spectrometrically, and total and free quercetin determined by HPLC are summarised in Fig. 2. According to the literature data about flavonoids in *Hypericum perforatum* (2), in all cases higher total flavonoid content than that of total quercetin is due to the non-selective spectrometric measurement including quercetin (free and in heterosydes) as well as luteolin and other flavonoids present in minor quantities. HPLC method, on the other hand, gives only the content of quercetin in the extracts.

Spectrometric determination of flavonoids in oil extracts

Satisfactory values of the recovery ranging from 83.8 to 97.5% (RSD = 22.4%, n = 10) were found for liquid-liquid extraction method. Analysis of total flavonoids expressed

as quercetin was made in 11 prepared oil extracts as well as in 4 commercial oils. The results are presented in Table III. Total flavonoids content in the extract B-3 (1.5 hours digestion) is 6.1 mg per 100 g, whereas for other extracts it ranges from 9.8–13.0 mg per 100 g with no significant variations depending on the duration of the extraction.

In the extracts prepared by exposure to sunlight for 15 and 40 days, 8.4–13.6 mg of total flavonoids per 100 g were found in the extract B-15 and 8.4–9.1 mg per 100 g in B-40. The results imply that flavonoids were extracted almost completely in first 15 days, and by further extraction no significant yield was achieved. High values obtained for flavonoids in some B-15 samples are probably due to the procedure imperfections (RSD = 22.4%).

By liquid-liquid procedure, total flavonoids were also determined in commercial samples of *Oleum hypericum*. An average of 12.8–13.8 mg per 100 g for C-1 and 8.3–10.2 mg per 100 g for C-4 has been found.

Aminopropyl columns suitable for preparation of samples containing heterocyclic compounds with oxygen as heteroatom and also phenolic groups bonded to the heterocycle were used. The values from 87.4–94.9% were obtained for recovery (RSD = 8.3%; n = 10). The results are given in Table III. The results for total flavonoid content in all oil extracts obtained by SPE method are slightly lower compared to those obtained with the former one. Our results for commercial samples, ranging from 7.5 to 13.7 mg per 100 g, are in good agreement with the results of Maisenbacher and Kovar (7).

Table III. Total flavonoids in oil extracts of Hypericum perforatum *determined spectrometrically*

Oil extract	Concentration (mg per 100 g)[a]	
	Classical procedure	Procedure with SPE
B-1	–	–
B-2	–	–
B-3	6.12 ± 0.62	–
B-4	12.25 ± 0.62	–
B-5	9.81 ± 0.54	–
B-6	12.99 ± 0.56	7.5 ± 0.27
B-7	10.98 ± 0.56	–
B-8	12.17 ± 1.74	8.40 ± 0.68
B-9	11.36 ± 0.88	1.42 ± 0.68
B-15	10.22 ± 2.35	9.50 ± 0.39
B-40	8.77 ± 0.35	8.86 ± 0.93
C-1	13.79 ± 0.44	12.82 ± 0.19
C-2	[b]	7.58 ± 0.10
C-3	[b]	7.47 ± 0.26
C-4	8.32 ± 0.27	10.20 ± 0.60

[a] Mean ± SD (n = 5). [b] On addition of $AlCl_3$ milk-like emulsion was formed.

CONCLUSION

Total flavonoid content in *Hyperici herba* (*Hypericum perforatum* L.) from Macedonia, was found to be 1.9% whereas the content of total quercetin was 1.3%. The results suggest that 70% ethanol is the most favorable solvent for extraction of heterosyde forms of flavonoids, whereas 50% ethanol gives the highest yield in extraction of aglycone forms of flavonoids. This applies both, to total flavonoids analysis and to quercetin analysis.

The highest yield of the extraction by olive oil was obtained after 3 hours of digestion at 100 °C. Total flavonoid content in this oil extracts was 13.0 mg in 100 g of extract.

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SAŽETAK

Određivanje ukupnih flavanoida i kvercetina u *Hyperici herba* i vodenim, vodeno-etanolnim i uljnim ekstraktima

SVETLANA KULEVANOVA, MARINA STEFOVA i TRAJČE STAFILOV

Istraživani su flavonoidi u *Hyperici herba* (*Hypericum perforatum* L., *Hypericaceae*) koje su skupljene u jugozapadnoj Makedoniji. Spektrometrijski je određeno da je sadržaj ukupnih flavonoida 1,9%. Njihov sadržaj i sadržaj ukupnih aglikona varirao je u vodenim, vodeno-etanolnim i etanolnim ekstraktima priređenim iz istog biljnog materijala. Najveća vrijednost ukupnih flavonoida (1,32 mg mL^{-1}) nađena je u ekstraktima sa 70%-tnim etanolom, a ukupnih aglikona (0,84 mg mL^{-1}) u ekstraktima sa 50%-tnim

etanolom. Sadržaj kvercetina određen je HPLC metodom i iznosio je 1,3%. Analogno flavonoidima, najveća vrijednost ukupnog kvercetina (0,68 mg mL^{-1}) pronađena je u ekstraktima sa 70%-tnim etanolom. Za ekstrakciju aglikona najpovoljniji je bio 50%-tni etanol (dobiveno je 0,24 mg mL^{-1} kvercetina). U ekstraktima s maslinovim uljem, najveći sadržaj ukupnih flavonoida dobiven je nakon trosatne digestije na 100 °C. Sadržaj ukupnih flavonoida u tim ektraktima bio je 13,0 mg po 100 g.

Ključne riječi: *Hypericum perforatum* L. (*Hypericaceae*), ekstrakt, flavonoidi, kvercetin, spektrometrija, HPLC

Institute of Pharmacognosy, Faculty of Pharmacy
Skopje, Republic of Macedonia

Institute of Chemistry, Faculty of Science
Skopje, Republic of Macedonia