Acta Poloniae Pharmaceutica – Drug Research, Vol. 58 No. 3 pp. 199–203, 2001

ISSN 0001-6837
Polish Pharmaceutical Society

NATURAL DRUGS

QUANTITATIVE DETERMINATION OF FLAVONOIDS IN THE FLOWERS AND LEAVES OF *PRUNUS SPINOSA L.*

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Abstract: The content of flavonoids in the flowers and leaves of *Prunus spinosa L.* was determined by spectrophotometric and RP–HPLC method. Determinations included hydrolysis of flavonoid glycosides in extracts from raw materials and then quantitative analysis of the obtained aglycones. Results were calculated for the content of glycosides and statistical analysis of the obtained data was performed.

Keywords: *Prunus spinosa L.*, flowers, leaves, flavonoids, quantitative determination, spectrophotometric and HPLC analysis.

Prunus spinosa L. (Rosaceae) – blackthorn, is a common plant in continental climate in the Northern Hemisphere (1). In Poland it can be found in lowlands and in lower parts of the mountains (2). Its therapeutic properties have been known for very long now (1). Flowers and leaves of blackthorn are used as diuretics, spasmolytics and anti–inflammatory agents (3, 4). Active components of these materials are flavonoids (3, 5), which have been determined in pharmacological studies (6). The fruits of blackthorn have been reported as an dietary product and also as styptic tannin, anti–inflammatory and antibacterial agents (3, 4).

According to literature, flowers and leaves of blackthorn contain complex of flavonoids, derivatives of flavonol: kaempferol, quercetin and their glycosides with arabinose, rhamnose and xylose (6–10). Moreover, the flowers contain A–type proantocyanidins (11), the leaves carotenoids and norisoprenoids (12, 13), and both materials contain phenolic acids (14).

Studies conducted at the Department of Pharmacognosy, Medical University of Łódź, confirmed the presence of kaempferol, quercetin and their heterosides in the flowers and leaves of blackthorn. In flowers, flavonoids are present mostly in the form of monoglycosides, mainly kaempferol and quercetin 3–O–arabinosides . Leaves are abundant in diglycosides, mainly kaempferol 3,7–O–dirhamnoside (15).

Extensive quantitative analysis of flavonoids in blackthorn has not been conducted so far. The content of flavonoids in the flowers of Romanian blackthorn population (1.16% of total flavone aglycones – quercetin) is the only value well known (7).

The aim of this work was quantitative determination of flavonoids in the flowers and leaves of blackthorn by spectrophotometric and HPLC methods.

EXPERIMENTAL

Plant material

The material for studies were flowers and leaves of *Prunus spinosa L.* collected from natural population in Piwniczna near Stary Sącz (1996, 1997, 1999) or purchased in the drugstore (1994, 1999, under the firm Zakład Zielarski S. Denel, Lubanów, Poland). The raw materials, obtained from natural population were dried in normal conditions, and then all materials were pulverized and sieved acc. to FP V (sieves 0.315 and 0.074 mm). The content of flavonoids was determined in materials, prepared in this way (marked as N–normal) and in materials, extracted previously with chloroform in Soxhlet apparatus and dried (marked as Sx–Soxhlet).

Voucher specimens were deposited at the Department of Pharmacognosy.

Quantification of flavonoids by spectrophotometric (Christ–Müller (16)) method

Instrumentation, reagents and solvents

The absorbance was measured by spectrophotometer Specol (Carl Zeiss Jena, GDR) at 425 nm.

The standard (quercetin) was purchased from Roth (see HPLC method). Reagents used: aluminium chloride ($AlCl_3 \times 6H_2O$), hydrochloric acid, urotropin and solvents: acetone, ethyl acetate, glacial acetic acid, methanol (P.O.Ch. Gliwice, Poland) were of analytical purity.

Calibration curve

A 50 mg of quercetin was dissolved in methanol (100 cm^3 volumetric flask) and 10 cm^3 of this solution was placed in a volumetric flask, followed by addition of 1 cm^3 of 0.5% urotropine water solution and dilution with methanol to 100 cm^3. To the volumetric flasks 12 different volumes of the obtained solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 6.5 cm^3, which means 0.025 to 0.325 mg of quercetin) were pipetted, then 1 cm^3 of 2% $AlCl_3$ solution in methanol–glacial acetic acid (19:1), 10 cm^3 of ethyl acetate and 0.75 cm^3 of glacial acetic acid were added and flasks were filled with methanol to 25 cm^3. After 45 min the absorbance was measured versus the solutions without $AlCl_3$.

Calibration curve of the dependence of absorbance on quercetin weight was described by equation: $y = 2.6224x + 0.0018$ (correlation coefficient $R = 0.9999$).

Analytical procedure

A 150 mg of raw material was heated under reflux for 30 min with 20 cm^3 of acetone, 2 cm^3 of 25% hydrochloric acid and 1 cm^3 of urotropine solution. The obtained extract was filtered through cotton wool, and the sediment with the cotton wool was heated twice for 10 min with 20 cm^3 of acetone. The extracts were mixed and diluted with acetone to 100 cm^3 in a volumetric flask. Then, 20 cm^3 of this solution was diluted with 20 cm^3 of water, extracted with 15 cm^3 of ethyl acetate and then, three times with 10 cm^3 of ethyl acetate. Organic phases were mixed and washed twice with 40 cm^3 of water, filtered to volumetric flask and diluted with ethyl acetate to 50 cm^3 . To four volumetric flasks 10 cm^3 of the obtained solution was added. Then, to three flasks 2 cm^3 of $AlCl_3$ solution was added and all four flasks were filled with methanol–glacial acetic acid (19:1) to 25 cm^3. After 45 min the absorbances were measured versus the solution without $AlCl_3$. For each of the studied raw materials, the procedure was repeated three times. The results are presented in Table 1.

Quantification of flavonoids by HPLC method

Instrumentation

The liquid chromatography equipment was made by Hewlett–Packard (1100 series system, Waldbronn, Germany) and consisted of a quaternary pump, autosampler, termostated column compartment, vacuum degasser, and diode–array detector. For instrument control, data acquisition and data analysis a Hewlett–Packard ChemStation for LC 3D system including single instrument Hewlett–Packard ChemStation software and Vectra color computer was used. The column used (Hypersil ODS, 125 × 4.0 mm, 5 µm) was purchased from Hewlett–Packard (Waldbronn, Germany).

Standards, reagents and solvents

The standards (quercetin and kaempferol, HPLC grade purity) were purchased from Roth (Basel, Switzerland). Methanol, water and ortophosphoric acid (Merck, Darmstadt, Germany) were of HPLC grade purity. Hydrochloric acid (P.O.Ch., Gliwice, Poland) was of analytical grade.

Chromatographic procedure

The mobile phase consisted of solvent A (0,5% water solution of ortophosphoric acid) and solvent B (methanol) with the elution profile as follows: 0–0.5 min, 40% B; 0.5–2.5 min, 40–70% B; 2.5–3.5 min, 70% B; 3.5–5.5 min, 70–40% B; 5.5–6.0 min, 40% B. The flow rate was 1.6 cm^3/min., the injection volume – 20 µl and detection was effected at 370 nm. Under these conditions quercetin and kaempferol eluted after 2.74 min and 3.09 min, respectively (Fig. 1A).

Calibration curves

Calibration curves were prepared for kaempferol and quercetin. A 1.65 mg of quercetin + 1.3 mg of kaempferol and 1.25 mg of quercetin + 1.05 mg of kaempferol were dissolved and diluted with methanol to 100 cm^3 in two volumetric flasks. By dilution, seven different solutions of quercetin were prepared (concentration from 0.78 to 6.25 µg/cm^3) and six solutions of kaempferol (concentration from 0.66 to 5.25 µg/cm^3). A 20 µl – volume of these solutions was injected into the HPLC system (three times). Calibration curves of dependence between peak area and concentration of standards were described by equations:

- $y = 362530x - 81355$ (correlation coefficient $R = 0.9995$) for quercetin
- $y = 471050x - 41642$ (correlation coefficient $R = 0.9985$) for kaempferol

Sample preparation

A 100 mg of raw material was heated under reflux for 1 hour with 6 cm^3 of 25% hydrochloric acid and 20 cm^3 of methanol. The obtained extract was filtered through cotton wool to volumetric flask. The sediment with cotton wool was then

Table 1. Contents (%) of flavonoids in flowers (F) and leaves (L) of *Prunus spinosa L.*

Sample	Spectrophotometric method				HPLC method			
	N		Sx		Sx			
	Σ Aglycones	Σ Glycosides***	Σ Aglycones	Σ Glycosides***	Quercetin	Kaempferol	Σ Aglycones	Σ Glycosides***
F 94**	3.33 (2.0)	4.70	3.27 (2.7)	4.61	1.39 (3.0)	1.16 (1.7)	2.55	3.60
F 04.96*	2.51 (0.4)	3.54	2.46 (1.9)	3.47	0.86 (2.3)	0.96 (2.4)	1.82	2.57
F 05.97*	3.58 (2.1)	5.05	3.42 (0.4)	4.82	1.32 (1.5)	1.32 (2.7)	2.64	3.72
F 99**	3.45 (2.5)	4.86	3.29 (3.9)	4.64	0.98 (1.1)	1.51 (5.4)	2.49	3.51
L 05.96*	2.16 (0.3)	4.19	1.65 (0.6)	3.20	0.41 (4.1)	0.92 (4.7)	1.33	2.58
L 05.97*	2.52 (1.6)	4.89	2.20 (1.2)	4.37	0.33 (3.6)	1.32 (1.5)	1.65	3.20
L 07.97*	1.98 (2.4)	3.84	1.50 (1.0)	2.99	0.37 (1.7)	0.76 (3.2)	1.13	2.19
L 10.97*	3.18 (2.0)	6.17	2.85 (6.4)	5.51	0.67 (1.8)	1.53 (4.2)	1.20	4.27
L 10.99*	2.21 (1.2)	4.29	1.73 (2.6)	3.36	0.38 (1.6)	0.68 (2.0)	1.06	2.06

Plant material from natural population* or purchased in the drugstore**.
Values in parentheses are relative standard deviations RSD (%) (n=3).
Calculation***: Σ Glycosides = f · Σ Aglycones; f=1.41 (for flowers) and f=1.94 (for leaves). The conversion factors (f) for the aglycones (for quercetin) are determined with the mean molecular weight of kaempferol and quercetin 3-O-arabinosides (for flowers) and with the molecular weight of kaempferol 3,7-O-dirhamnoside (for leaves).

heated twice with 20 cm^3 of methanol for 10 min. Mixed extracts were diluted with methanol to 100 cm^3. A part of the obtained solution was filtered through a syringe filter PTFE 13 mm, 2 μm (Whatman, England). Then, 2.5 or 5.0 cm^3 of the filtrate (3 samples) were diluted with methanol in a volumetric flask to 10 cm^3 and 20 μl of the each of so prepared solutions was injected into the HPLC system. The results are presented at Fig. 1B, 1C and in Table 1.

RESULTS AND DISCUSSION

A method frequently employed to determine the content of flavonoids in plant raw materials, is the spectrophotometric method with aluminium chloride (Christ–Müller method). It is used to determine the content of flavonoids in the form of aglycones, after hydrolysis of glycoside bonds in these compounds. This method is especially useful in materials, which contain flavonols, easy to hydrolyse and well soluble in ethyl acetate. However, this method (or its modifications) included in several Pharmacopoeias (as well in FP V), is not specific and allows to determine only total content of flavonoids in plant material. For this reason, we have decided to apply, additionally, HPLC method in order to analyse precisely quantitative content of quercetin and kaempferol in the studied material.

The content of flavonoids was determined in plant raw materials, prepared in the standard way according to FP V (N) and in raw materials, initially purified by extraction with chloroform (Sx). This enabled comparison between both methods. For HPLC, due to equipment requirements, the studied samples had to be purified beforehand.

The total flavonoids content in the flowers of blackthorn determined by spectrophotometry was, for quercetin: 2.51 ÷ 3.58% for N materials and 2.46 ÷ 3.42% for Sx materials. Calculated for monosides, main components of these materials, this content was 3.54 ÷ 5.05% (N) and 3.47 ÷ 4.82% (Sx). It is about twice as high as in the flowers obtained from Romania (7). The content of flavonoids in the leaves, calculated for quercetin, was 1.98 ÷ 3.18% (N) and 1.50 ÷ 2.85% (Sx) and calculated for glycosides 3.84 ÷ 6.17% (N) and 2.99 ÷ 5.51% (Sx).

In recent years, HPLC was found as the preferred method for the determination of the content of flavonoids as its results are more precise and acurate. HPLC determinations have also been suggested for hydrolysed extracts from raw materials, which significantly shortens the time of analysis (17–19).

In this work we have evolved conditions for hydrolysis of raw materials which contain, apart

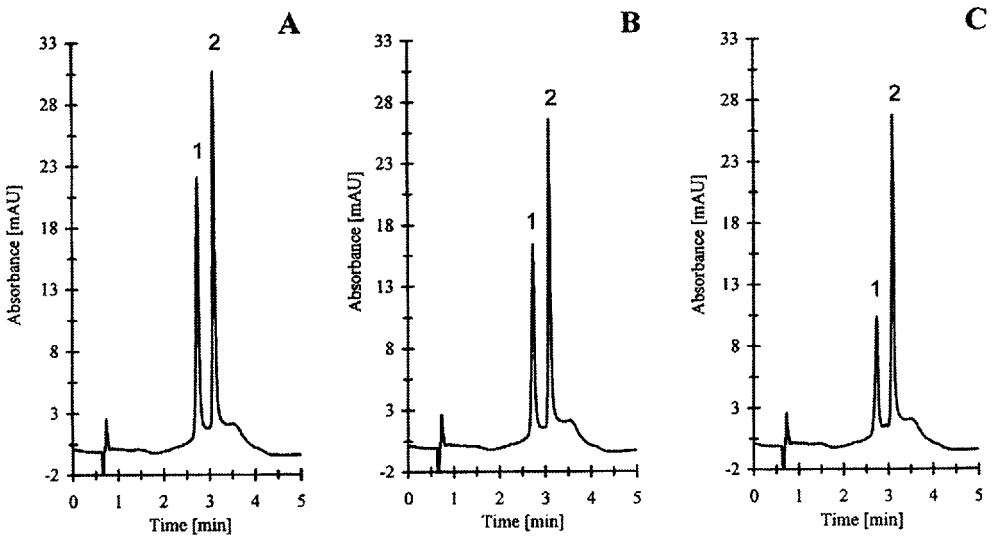

Figure 1. HPLC chromatogram of quercetin (1) and kaempferol (2). A: standard methanol solution, both (1) and (2): 0.057 μg in peak. B: real sample – extract from flowers (collected 05.1997), both (1) and (2): 0.050 μg in peak. C: real sample – extract from leaves (collected 07.1997), (1): 0.027 μg and (2): 0.050 μg in peak. Low limit of detection: (1): 0.024 μg/cm^3 and (2): 0.017 μg/cm^3 (based on signal to noise ratio of 3:1). Low limit of quantitation: (1): 0.080 μg/cm^3 and (2): 0.057 μg/cm^3 (based on signal to noise ratio of 10:1).

from flavonols, proanthocyanidins and conditions for the separation of kaempferol and quercetin on RP–18 column. The elaborated procedure, employing gradient of methanol in 0.5% orthophosphoric acid (v/v), enabled performing of a single analysis in 5.5 min (quercetin and kaempferol eluted after 2.74 min and 3.09 min, respectively). This procedure, applying more intensive gradient elution, constitutes modification of the earlier described HPLC method (17), according to which quercetin and kaempferol eluted after 5.46 min and 8.83 min, respectively.

The total content of aglycones determined in this way in the flowers of blackthorn was 1.82 ÷ 2.64% (calculated for glycosides 2.57 ÷ 3.72%). Leaves contained 1.06 ÷ 1.65% of aglycones, which corresponds to 2.06 ÷ 4.27% of glycosides. Moreover, kaempferol and quercetin were present in the flowers in proportion about 1:1 and in the leaves, about 2.4:1.

Differences, observed between the results obtained by spectrophotometric and HPLC methods, showed significant error in the spectrophotometric method. This error results from the presence of other compounds in the materials, which can react with aluminium chloride.

CONCLUSIONS

Flowers and leaves of *Prunus spinosa L.* are a rich source of flavonoid compounds, the content of which in the studied materials is the same or higher than in many valuable therapeutic raw materials, such as Sambuci flos or Betulae folium. As pharmacological studies confirmed, biological activity of flavonoid compounds present in blackthorn, flowers and leaves of this plant, which is easily accessible in our climate, is worthy of wider interest. In particular, high content of flavonoids in the leaves of blackthorn, so far used only in folk medicine, is interesting as they are easier and cheaper to obtain than the flowers.

The proposed method of quantitative RP–HPLC analysis of aglycones can be recommended as an easy and quick method for standardisation of flavonoid raw materials and therapeutic preparations containing derivatives of flavonols.

Acknowledgements

This work has been financed by Medical University of Łódź from an internal grant No. 502–13–442.

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Received: 10.10.2000