ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXII, N 4, 15 SECTIO DDD 2009

Department of Pharmaceutical Botany, Medical University of Lublin

ANNA SOKOŁOWSKA-KRZACZEK, KATARZYNA SZEWCZYK, ZOFIA BAWÓŁ

Determination of free and bound phenolic acids in leaves and fruits of Pyracantha coccinea (L.) Roem. (Rosaceae)

Oznaczenie wolnych i związanych kwasów fenolowych w liściach i owocach *Pyracantha coccinea* (L.) Roem. (*Rosaceae*)

Pyracantha coccinea (L.) Roem. (*Crataegus pyracantha* Borlch., *Cotoneaster pyracantha* (L.) Spach., firethorn) [14] is an urban-tolerant shrub with spiny branches, broadleaf evergreen dense foliage, spire-like upper branches, and orange autumn fruits. *Pyracantha* grows well in slightly-acidic, well-drained soils. It is fairly drought-tolerant once established and does best when planted in full sun or shifting shade [5, 6]. It grows easily in both urban and rural areas of various geographical ranges. *P. coccinea* is distributed especially North, Central and South Anatolia in Turkey, South Europe, Crimea, Caucasia, North-West Iran in the world. Its distribution has association with the climate, because *Pyracantha* prefers warm and tender climate. This genus is also cultivated for its ornamental berries [5, 14].

Numerous studies show that *P. coccinea* could be used as a biomonitor for heavy metals (like Zn, Cd, Pb), especially with its branches and leaves. Firethorn is well-known for its therapeutic activities. Previous phytochemical studies included examination of different parts of *P. coccinea* in different phases of growth. In this species, flavonoids, triterpenes, tanins, carotenoids, coumarins and sterols were detected [1, 2]. Its fruits were used as diuretic, cardiac and tonic agents in folk medicine. Chalcone and lignan glucosides isolated from this plant have antibacterial, antifungal and hypotensive properties [3, 9]. Phenolic acids may be responsible for hypotensive and diuretic effects of the extracts from *P. coccinea*.

The aim of this paper was identification of phenolic acids in the fruits and the leaves from *Pyracantha coccinea* (L.) Roem. by chromatographic methods.

MATERIAL AND METHODS

Plant material. The plant material for research were the fruits and leaves of *Pyracantha coccinea* collected in the Botanical Garden in Lublin. The leaves were collected in June and the fruits in September. All material was cleaned and inspected to remove damaged and diseased parts. The leaves were dried in normal conditions and the fruits were dried at 45–50˚C. All raw material was pulverized and sieved according to Polish Pharmacopoeia VI [8]. 180g of the fruits and 120g of the leaves were used in the study.

Extraction and isolation. The dried plant materials were previously extracted with

petroleum ether in Soxhlet apparatus (60h, 45–60˚C), and then were extracted twice with boiling 80% methanol and once with 60% boiling methanol. After evaporation of the solvents, the residues were eluted with hot water portions and after 24h the aqueous fractions were filtered. After purification, phenolic acids fractions were isolated in a typical way for this group of compounds. The processes of acidic hydrolysis of phenolic acids associated with sugar were performed according to Schmidtlein and Herrman (1975) and Świątek and Dombrowicz (1984). Alkaline hydrolyses were performed with $Ba(OH)_2$ in reducing medium $NaBH_4$ [11, 15, 17]. The obtained fractions of free phenolic acids (A) and bound phenolic acids released by both, first alkaline and next acidic hydrolysis (B), were dissolved in ethanol and analyzed by TLC and HPLC.

Qualitative chromatography (1D-and 2D-TLC). Two-dimensional thin layer chromatography (2D-TLC) was performed on 100x100x0.1 mm cellulose plates (E. Merck, Darmstadt). After spotting of the phenolic acids standard solutions or plant extracts, the plates were developed "face down" in a DS horizontal chamber [7] using the following mobile phases [16]: I direction – U_1 : benzene – methanol – acetic acid – acetonitryle (80:10:5:5, v/v/v/v), II direction – U_2 : sodium formate – formic acid – water (10:1:200, v/v/v). To avoid eluent demixion, the plates were conditioned for about 5 minutes above vapours of U_3 – benzene – methanol – acetic acid (90:5:5, v/v/v) and then developed in the first direction. After drying, the chromatograms were observed in UV light (λ=366nm) before and after treatment with ammonia vapours. Visualization of phenolic acids by derivatisation was performed by spraying with one of the following reagents [10, 12]: W_1 – diazotized sulphanilic acid in 10% sodium carbonate solution; W_2 – diazotized p-nitroaniline in 20% sodium carbonate solution; W_3 – 2% aqueous solution of ferric chloride. After derivatisation, the chromatograms were observed in daylight. Phenolic acids were identified on the basis of a comparison of analyzed compounds with authentic standards.

The one-dimensional chromatography (1D–TLC) was performed on 100x200x0.1 mm silica gel plates (Kieselgel, Merck) for identifying phenolic acids occurred near or on starting-line. These standards. The obtained results of 1D-and 2D-TLC analyses are shown in Table 1.

Table 1. The occurrence of phenolic acids in *Pyracantha coccinea* (L.) Roem

No.	Phenolic acids	Leaves		Fruits	
		A	B	A	B
1	ellagic	+	-	+	-
2	gallic	-	+	-	-
3	protocatechuic	+	+	+	+
4	homoprotocatechuic	-	+	-	-
5	caffeic (trans + cis)	+	+	-	+
6	gentisic	-	+	-	+
7	p-hydroxybenzoic	-	+	-	+
8	p-coumaric	+	+	-	-
9	syringic	-	-	+	-
10	vanillic	+	+	+	+
11	ferrulic (trans + cis)	-	+	-	-
12	salicylic	+	+	-	-
13	α – resorcylic	+	-	-	-
14	γ - resorcylic	+	+	-	+

+ detectable, - not detectable, A – free phenolic acids fraction, B – bound phenolic acids fractions (liberated after acidic and alkaline hydrolysis)

chromatograms were developed in the U_4-toluene-n-propanol-formic acid-water (15:18:10:5, v/v/v/v) mobile phase. After drying in normal conditions, the chromatograms were visualizated by derivatisation with W_3 (2% aq. solution of ferric chloride).

Phenolic acids were identified on the basis of a comparison of the colors (before and after derivatisation), spot location, and R_F values of the analyzed compounds with those of authentic

Semi-quantitative chromatography (RP – HPLC). The fractions isolated from leaves (A, B) and fruits (A, B) were analyzed by HPLC. Before analyses the samples were filtrated using Iso-Disc (25mm x 0.45µm) filters. The RP-HPLC analysis was performed using Knauer (Germany) liquid chromatograph equipped with a K-1001 pump, a K-2001 UV detector operating at 254 nm, 20µl sample injector (Rheodyne, Cocati, CA, USA) and a stainless-steel column packed with Hypersil ODS (200mm length; 4.6mm in diameter and 5µm thickness of granules). The flow rate was 0.5 ml/min. The isocratic mobile phase consisted of methanol-water (25:75, v/v) with 1% v/v acetic acid was used. Determination of the content of investigated compounds was made on the basis of linear dependence between peak areas and concentration. The external standard method was used. The chromatograms were recorded and the amounts of phenolic acids were determined with Eurochrom 2000 software. The results of RP-HPLC analyses are given in Table 2. The retention time values of standards and phenolics isolated from leaves and fruits of *P. coccinea* are shown in Tab. 3.

Table 2. The content of major phenolic acids in *Pyracantha coccinea* (L.) Roem.

No.	Phenolic acids	Content of phenolic acids in mg/100g (dry weight)					
		leaves			fruits		
		free (A)	bound (B)	total (A+B)	free (A)	bound (B)	total (A+B)
1	protocatechuic	17.38	2.89	20.27	0.2	2.06	2.26
2	p-hydroxybenzoic	-	0.17	0.17	-	0.002	0.002
3	vanillic	0.002	3.28	3.282	0.002	0.18	0.182
4	caffeic	1.29	6.71	8	-	5.87	5.87
5	syringic	-	-	-	0.01	-	0.01
6	p-coumaric	0.17	3.04	3.21	-	-	-
7	ferullic	-	0.12	0.12	-	-	-

A – free phenolic acids fraction, B – bound phenolic acids fractions (released after acidic and alkaline hydrolysis)

Table 3. Retention time values of standards and phenolics isolated from leaves and fruits of *P. coccinea*

		Retention times values t_R (min)				
No.	Phenolic acids	standard	leaves (A)	leaves (B)	fruits (A)	fruits (B)
1	protocatechuic	4.173	4.233	4.050	3.987	4.183
2	p-hydroxybenzoic	7.010	-	6.800	-	7.230
3	vanillic	7.945	8.030	8.133	7.890	8.347
4	caffeic	8.727	8.517	8.800	-	9.133
5	syringic	9.833	-	-	10.145	-
6	p-coumaric	14.780	15.893	16.093	-	-
7	ferullic	19.510	-	19.983	-	-

A – free phenolic acids fraction, B – bound phenolic acids fractions (liberated after acidic and alkaline hydrolysis)

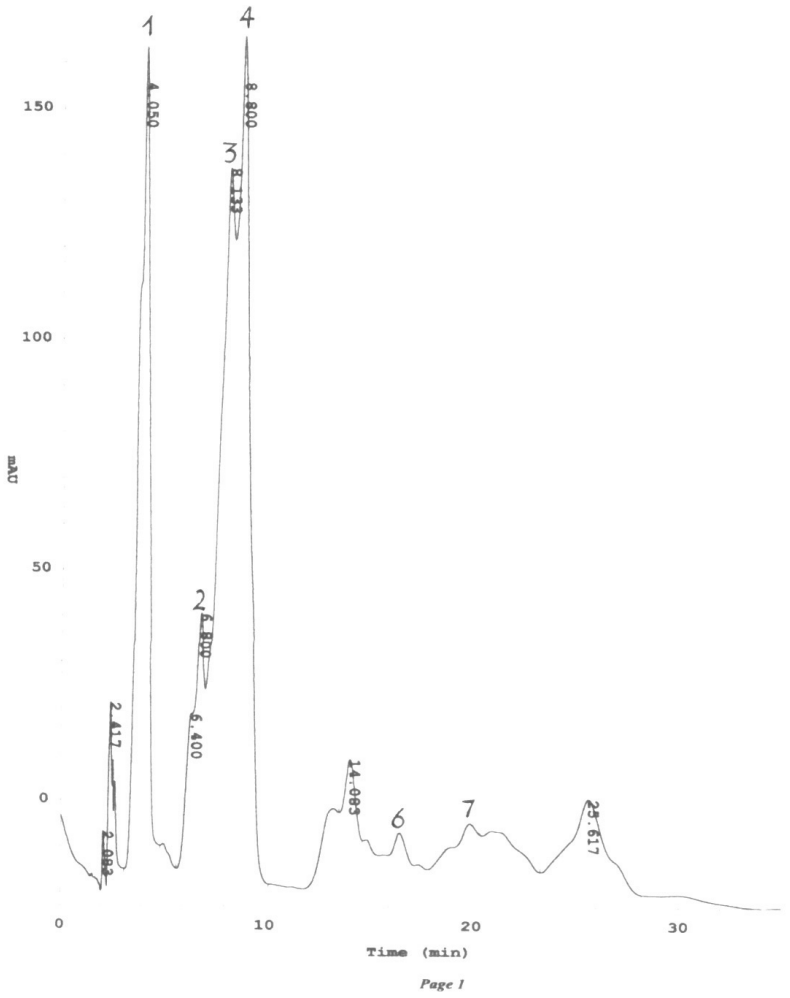

Fig. 1. The HPLC chromatogram of the bound phenolic acids fraction (Fr. B) from the leaves of *Pyracantha coccinea*. The identification numbers of peaks refer to compounds listed in Table 2

RESULTS AND DISCUSSION

The fractions of the free and bound phenolic acids prepared from water-methanol extracts of leaves and fruits of *P. coccinea*, were analysed by chromatographic methods like 2D-TLC and RP-HPLC. Fourteen compounds were identified in total: ellagic, gallic, protocatechuic, homoprotocatechuic, caffeic (trans+cis), gentisic, p-hydroxybenzoic, p-coumaric, syringic, vannilic, ferulic (trans+cis), salicylic, α-resorcylic and γ-resorcylic. They are described for the first time in the investigated plant. Most of the phenolic acids occurred both in free and bound forms. The leaves are the richest in the phenolic acids part of the plant. In the leaves, thirteen phenolic acids were identified. Most phenolic acids in the fruits appeared to occur in the bound form only. The ellagic, protocatechuic, caffeic (cis and trans), gentisic, p-hydroxybenzoic, vanillic and α-resorcyllic acids were common in both plant materials. Most characteristic acids of *Pyracantha coccinea* turned out to be protocatechuic and

vanillic acids, which occur in all fractions of both researched raw materials. The leaves as well as fruits showed the presence phenolic acids, for example syringic acid was identified only in fruits in the fraction A.

The RP-HPLC method enabled estimation of the content of major phenolic acids in all obtained fractions. They are quite diverse and range from 0.002 up to 17.38mg/100g of the dry material. A large increase of the quantities of acids in the fractions obtained by hydrolyses was observed. For example, the amount of vanillic acid released after both alkaline and acidic hydrolyses increased from 0.002mg to 3.23mg/100g of dry leaves of *P. coccinea*. Protocatechuic acid is a remarkably dominating constituent of both plant materials (17.38mg% – leaves (A); 2.89mg% – leaves (B); 0.204mg% – fruits (A); 2.06mg% – fruits (B)).

Most of the identified phenolic acids show a certain pharmacological activity. A attention should be paid especially to the acids present in larger amounts in *P. coccinea*, such as protocatechuic, caffeic and vanillic, which are known as having antimicrobial, antiphlogistic and fungicidal properties [4, 13].

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SUMMARY

The analysis of the fractions of free and liberated by hydrolysis phenolic acids in the fruits and the leaves of *Pyracantha coccinea* was conducted by TLC and RP-HPLC methods. In the fruits eight and in the leaves thirteen phenolic acids were identified. By means of the RP-HPLC method the content of major acids was estimated, which ranges from 0.002 up to 17.38mg/100g of the dry material. Protocatechuic acid is a remarkably dominating constituent of both plant materials.

STRESZCZENIE

Za pomocą metod TLC i RP-HPLC przeprowadzono analizę frakcji wolnych i uwolnionych po hydrolizie kwasów fenolowych w owocach i liściach *Pyracantha coccinea*. W owocach zidentyfikowano 8, a w liściach 13 kwasów fenolowych. Przy pomocy RP-HPLC oszacowano zawartość głównych kwasów, która kształtowała się na poziomie 0,002–17,38mg/100g suchego surowca. Dominującym składnikiem w obu badanych surowcach był kwas protokatechowy.