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COMPARATIVE ANALYSIS OF PHENOLIC ACIDS IN MISTLETOE PLANTS FROM VARIOUS HOSTS

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Abstract: Phenolic acids present in mistletoe plants collected from various hosts were analysed with the use of HPLC. The following numbers of compounds were found in the mistletoe plant material gathered from respective hosts: *Sorbus aucuparia*– 12 compounds; *Acer plantanoides*– 14 compounds; *Malus domestica, Pyrus communis* and *Populus nigra* – 13 compounds each; *Quercus robur*– 15 compounds. Altogether 21 phenolic acids were chromatographically identified in the tested material. The compounds were either free or combined as esters or glycosides. Comparative chromatography revealed qualitative differences in the investigated compounds between the various plant materials. For example o–coumaric acid was only found in mistletoe hosted by *Quercus robur*. Digallic acid was only found in the plant material hosted by *Acer plantanoides*. Qualitative and quantitative composition of mistletoes hosted by *Malus domestica* and *Pyrus communis* showed considerable similarities as far as phenolic acids were concerned. Moreover, vanillic acid, absent in all other batches of plant material, seemed to be characteristic of the above mistletoes. Quantitative HPLC analysis demonstrated a considerable content of salicylic acid (39.55 mg%) in mistletoe hosted by *Sorbus aucuparia*. Apart from the above material, this compound was only present in small quantities in plants hosted by *Populus nigra* (15.63 mg%) and *Quercus robur* (2.63 mg%).

Keywords: *Viscum album* L, phenolic acids, host plants – comparison, RP–HPLC analysis.

Viscum album L. is an epiphytic parasite of coniferous and deciduous trees, believed since ancient Greek and Roman times, to have medicinal properties (1).

Modern phytochemical research showed that mistletoe twigs and leaves are a rich source of numerous pharmacologically active compounds. The plant was found to contain, among others: peptide compounds (including a cytotoxic visco–toxine), lectins, a number of amino acids, flavonoids, phenolic acids, triterpenoids and many other compounds (2,3,4). In modern medical practice, mistletoe preparations are primarily used for their anticancer properties, ability to lower blood pressure and stimulate the immune system (4,5,6).

It is widely appreciated that the chemical composition of mistletoe is not stable and depends not only on the biosynthesis but also on the type of host plant and growing conditions, such as ambient temperature, carbon dioxide concentration and season of the year (3,7). Therefore a number of difficulties occur in standardising medical preparations made of mistletoe and in obtaining secondary metabolites from the plant material. Attempts are also made to use specific types of compounds produced by mistletoe to identify plants growing on a particular host. So far none of the groups of secondary metabolites or specific compounds were identified as possible chemical–taxonomic factors.

This paper aims at full qualitative and quantitative analysis of phenolic acids in the leaves and twigs of mistletoe from various hosts. The above compounds in *Viscum album* have only been investigated partially, as part of analysis of other chemical groups (8,9,10). Moreover, the research quoted concentrated only on qualitative analysis of free phenolic acids. It also was not shown, whether there is a direct relation between qualitative and quantitative content of phenolic acids in the mistletoe and the type of host plant.

The above issues will be investigated in this paper. In addition, determining a full qualitative and quantitative composition of phenolic acids may be important for the interpretation of multidirectional effects of mistletoe, including its immunostimulating properties, as phenolic acids are often considered for their role in regulating immunological processes (11,12).

EXPERIMENTAL

Plant material

Twigs and leaves o *Viscum album* L. (female plants) gathered in the autumn 1997 in the Gdańsk coastal area underwent phytochemical analysis. The plant material was obtained from the following hosts: *Sorbus aucuparia* L., *Malus domestica* L., *Pyrus communis* L., *Populus nigra* L., *Acer plantanoides* L., and *Quercus robur* L.

A voucher specimen of the plants was deposited in the herbarium of the Medicinal Plant Garden, Medical University of Gdańsk.

Extraction and isolation of the phenolic acids fraction

Dried and finely powdered plant material (10 g from each host) was exhaustively extracted with methanol by heating under a reflux condenser for 90 min. The extracts were then concentrated, diluted with water, filtered, purified and partitioned into fractions according to a general procedure elaborated for phenolic acids (13). As a result, fractions of free phenolic acids (A) were obtained. Each of the aqueous phases remaining after extraction was divided into two portions. The first one was subjected to acid hydrolysis (1N HCl) and marked (B). The second one, marked (C), was subjected to alkaline hydrolysis (1N KOH in a boiling water bath for 15 min.). Conditions of phenolic acid hydrolysis were in accordance with the published procedure (13).

Chromatography

Equipment

The HPLC system used was from Knauer (Berlin, Germany) and consisted of a pump, Model 64–00 and a Model 87–00–UV detector, equipped with a Model 7125 injection valve (Rheodine, Cotati, CA, USA) with a 20 µl sample loop, under computer control (Knauer HPLC, version 211a). Phenolic acids were separated on a LiChrospher RP–18 (5 µm) column (250 × 4 mm. I.D.Merck, Darmstadt, Germany).

Reagents

The organic solvents were of HPLC grade (Merck's acetonitrile, J.T. Baker's phosphoric acid). Redistilled water was used. After preparation, the mobile phase was filtered through 0.49 nm filter (J.T. Baker, Phillipsburg, NY, USA).

Elution

Phenolic acids were separated by isocratic elution using the mobile phase F_I: (water:acetonitrile:phosphoric acid 85:13.8:1.2). Elution was carried out at room temperature at a flow rate of 1.0 ml/min and UV detection at 320 nm (sensitivity 0.008 AUFS).

Reference compounds

The following phenolic acids *viz.*, trans–cinnamic, o–coumaric, p–coumaric, m–coumaric, caffeic, ferulic, syringic, sinapic, chlorogenic, protocatechuic, ellagic, digallic (Merck, Germany); p–hydroxybenzoic, isochlorogenic, genistic, isoferulic, rosmarinic, veratric, vanillic and salicylic (Extrasynthese, France) were used as standard compounds.

Calibration

Stock solution of chlorogenic acid was prepared by dissolving 2 mg of this compound in 10 ml of methanol. The volumes injected (20 µl) corresponded to amounts of this compound in the range of 1–4 µg. Calibration graphs were obtained by plotting the peak area (y) against the concentration of standard solutions (x).

Regression equation for chlorogenic acid: $y=2.401x-0.173$; correlation coefficient $r = 0.999$.

Sample preparation

The methanolic extracts for quantitative HPLC analysis were prepared from 10g of dry plant material, according to the procedure described above. All phenolic acid fractions (A, B, C) were evaporated at a reduced pressure to form syrup–like residues and these were, in turn, diluted with 25 ml of methanol (Merck). The solutions were filtered through 0.45 µm filters (J.T. Baker) and injected into the chromatographic column.

RESULTS AND DISCUSSION

Comparative analysis of *Viscum album* L. from various hosts was carried out as far as phenolic acids were concerned. In order to identify possible variations of the content of phenolic acids in different batches of the plant material, female mistletoe plants from the following hosts: *Sorbus aucuparia* L., *Malus domestica* L., *Pyrus communis* L., *Populus nigra* L., *Acer plantanoides* L., and *Quercus robur* L. gathered in the Gdańsk coastal area were selected for research.

Phytochemical research of phenolic acids covered both the free compounds and the combined ones into esters and glycosides. The HPLC method was used to identify individual compounds and their quantity in the plant material, using purified

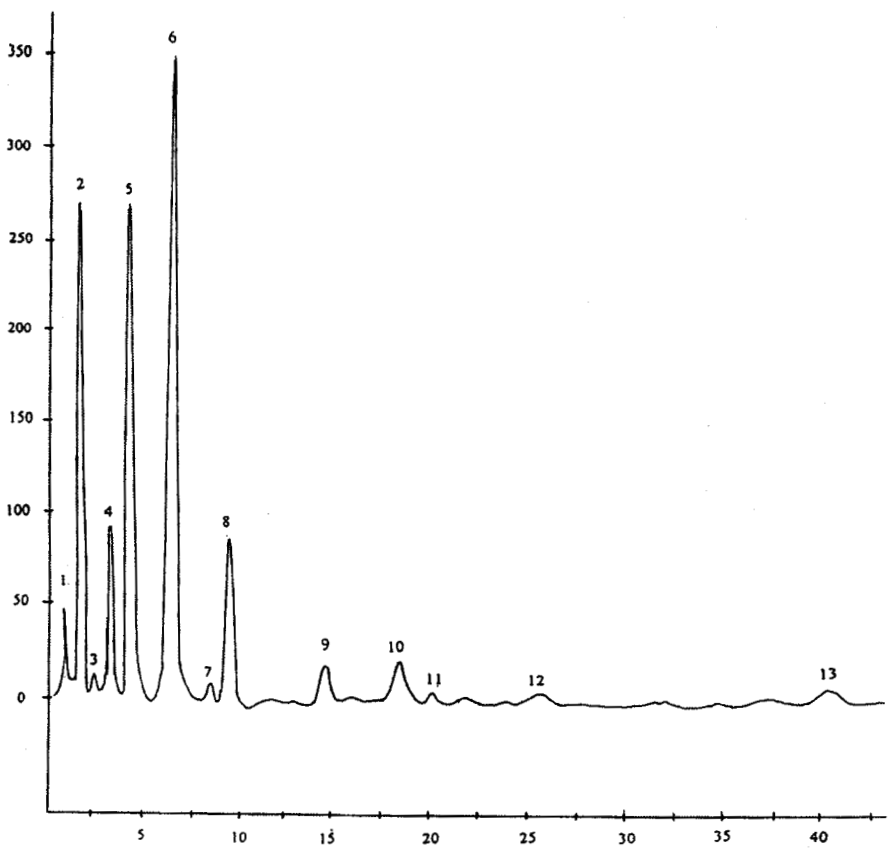
extracts prior to and after acid and alkaline hydrolysis.

In order to carry out chromatographic analysis, a full set of phenolic acids was extracted from the various batches of the plant material and then purified using a modified Świątek method (13,14,15). The modification consisted in that after the methanol extracts were preliminarily purified from lipophilic ballast substances, they were precipitated with acetone in order to remove polysaccharides which hinder chromatographic identification of phenolic acids. The Świątek method was also used to carry out acid and alkaline hydrolysis of the extracts. As a result the hydrolysis processes produced compounds which originally were glycosides (acid hydrolysis) or esters (alkaline hydrolysis). This procedure made it possible to carry out chromatographic analysis of the full set of phenolic acids present in the studied plant material.

Chromatographic system F_1 (see Experimental) was used to carry out qualitative and quantitative analysis. The system allowed a full separation of the rich set of phenolic acids in the analysed mistletoe plants (Figure 1). In general, 21 phenolic acids, which were PCA derivatives, HCA derivatives or depsides, were identified (Table 1). Phenolic acids were present as free compounds (fraction A), or combined as glycosides (fraction B) or esters (fraction C). (Table 1).

In case of *Viscum album* hosted by *Sorbus aucuparia*, a 12 compound set of phenolic acids was identified. Four phenolic acids, i.e. trans–cinnamic, gallic, caffeic and ferulic, were identified in all the tested fractions (A, B and C). Salicylic and chlorogenic acids are present in mistletoe hosted by *Sorbus aucuparia* as free compounds and also combined with sugars (fraction B) (Table 1). It is worth noting that protocatechuic acid present in this plant material, is only represented in an ester form (fraction C).

Quantitative HPLC analysis of the above phenolic acid fraction showed that the dominating



Peaks: 1 – trans–cinnamic acid, 2 – gallic acid; 3 – p–hydroxybenzoic acid, 4 – protocatechuic acid, 5 – isochlorogenic acid, 6–chlorogenic acid, 7 – caffeic acid, 8 – gentisic acid, 9 – rosmarinic acid, 10 – p–coumaric acid, 11 – m–coumaric acid, 12 – ferulic acid, 13 – salicylic acid.

Figure 1. A chromatogram of phenolic acid fraction (free forms) from *Viscum album* hosted by *Quercus robus* L.

Table 1. The occurence of phenolic acids in mistletoe plants from various hosts.

Comp. No.	Phenolic acids	HPLC analysis R_t	*Sorbus aucuparia L.*			*Acer plantanoides L.*			*Malus domestica L.*			*Populus nigra L.*			*Quercus robur L.*			*Pyrus communis L.*		
			A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1.	trans–cinnamic	2.07	+	+	+	+	+	+	+	–	traces	+	+	+	+	+	+	+	–	+
2.	gallic	2.51	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.	p–hydroxybenzoic	3.08	–	–	–	+	+	–	–	–	–	–	traces	–	traces	traces	–	–	–	–
4.	protocatechuic	4.40	–	–	+	+	–	+	–	–	+	+	–	+	+	–	+	–	–	+
5.	isochlorogenic	5.61	–	–	–	+	+	–	–	–	–	+	+	–	+	+	–	–	–	–
6.	chlorogenic	6.11	+	+	–	+	+	–	+	+	–	+	+	–	+	+	–	+	+	–
7.	caffeic	9.11	+	+	+	+	+	+	+	+	+	+	+	+	+	traces	–	+	+	+
8.	gentisic	9.90	+	–	+	+	+	+	–	–	+	+	+	traces	+	+	+	–	–	+
9.	syringic	10.37	+	–	–	+	–	–	+	–	+	traces	–	–	–	–	–	+	–	+
10.	isoferulic	12.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
11.	rosmarinic	15.35	–	–	–	+	–	–	+	–	–	–	–	–	+	–	–	+	–	–
12.	p–coumaric	19.55	+	–	+	+	+	+	+	–	+	+	–	+	+	+	–	+	–	+
13.	m–coumaric	21.51	–	–	–	–	–	–	+	–	–	–	–	–	traces	–	–	+	–	–
14.	veratric	22.19	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–
15.	ferulic	24.94	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–
16.	sinapic	27.17	–	–	–	–	–	–	traces	–	–	–	–	–	–	–	–	+	–	–
17.	vanillic	27.40	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+	–
18.	digallic	30.01	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19.	salicylic	39.17	+	+	–	–	–	–	–	–	–	+	+	–	+	+	–	–	–	–
20.	ellagic	43.95	+	–	–	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–
21.	o–coumaric	44.01	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

A – fraction containing free phenolic acids; B – fraction containing acids liberated by mild acid hydrolysis; C – fraction containing acids liberated by alkaline acid hydrolysis

Table 2. The content of phenolic acids in mistletoes hosted by *Sorbus aucuparia L.* and *Acer plantanoides L.* (mg% in dry plant material)

Comp. No.	Phenolic acids	*Sorbus aucuparia L.*			*Acer plantanoides L.*		
		A	B	C	A	B	C
1.	trans–cinnamic	4.53	9.60	4.72	1.17	5.77	2.86
2.	gallic	5.74	11.67	6.29	0.25	4.15	1.11
3.	p–hydroxybenzoic	–	–	–	0.82	1.24	–
4.	protocatechuic	–	–	0.03	7.27	–	0.09
5.	isochlorogenic	–	–	–	5.75	6.03	–
6.	chlorogenic	13.13	14.00	–	13.06	13.92	–
7.	caffeic	9.91	12.74	0.27	2.63	2.92	0.08
8.	gentisic	9.52	–	0.13	15.36	11.20	4.2
9.	syringic	8.37	–	–	7.78	–	–
10.	isoferulic	–	–	–	–	–	–
11.	rosmarinic	–	–	–	0.65	–	–
12.	p–coumaric	4.21	–	0.72	5.28	7.36	3.06
13.	m–coumaric	–	–	–	–	–	–
14.	veratric	0.78	–	–	–	–	–
15.	ferulic	9.21	4.44	1.79	0.32	0.78	5.23
16.	sinapic	–	–	–	–	–	–
17.	vanillic	–	–	–	–	–	–
18.	digallic	–	–	–	2.58	–	–
19.	salicylic	18.21	21.34	–	–	–	–
20.	ellagic	17.53	–	–	6.68	4.93	–
21.	o–coumaric	–	–	–	–	–	–

For explanation: A,B,C see Table 1

compound in *Viscum album* hosted by *Sorbus aucuparia* is salicylic acid (Table 2). This compound is present as the free acid in the amount of 18.21 mg% and as glycoside – 21.34 mg%. Such a large quantity of salicylic acid was not present in any of the other analysed plant materials and seemed typical of this specific mistletoe.

The mistletoe hosted by *Acer plantanoides*, had a 14 – element set of phenolic acids. Six phenolic acids, i.e. trans–cinnamic, gallic, caffeic, ferulic, gentisic and p–coumaric acids were present in the free acid fraction (A) and the fractions obtained through hydrolysis (fractions B and C) (Table 1). The following phenolic acids were identified here, which were not present in mistletoe hosted by *Sorbus aucuparia*: p–hydroxybenzoic, isochlorogenic, rosmarinic and digallic. While the first two were both present as free compounds and combined as glycosides, rosmarinic and digallic acids were only present as free. The presence of digallic acid (2.58 mg%) in mistletoe hosted by *Acer plantanoides* is worth noting as the compound was not present in any other batches of plant material and seems typical of this particular mistletoe (Table 2). Moreover, salicylic acid was not identified in this plant, even though it was the main component of the set of phenolic acids in *Viscum album* hosted by *Sorbus aucuparia*. In case of plant material collected from *Acer plantanoides*, gentisic acid was a dominant compound in the set of phenolic acids and it was present in the free fraction A in the amount of 15.36 mg% and fractions B and C in the amount of 11.20 mg% and 4.2 mg%, respectively.

HPLC chromatography of mistletoe hosted by *Malus domestica* and P*yrus communis* showed significant similarities between both batches of plant material in terms of phenolic acids present. The analysed mistletoes contained an identical 13–element set of phenolic acids, free and combined. Differences related only to different quantities of individual compounds (Table 3).

The dominating element was rosmarinic acid, present only in the free form (fraction A) in the amount of 17.48 mg% (*Malus domestica*) and 14.32 mg% (*Pyrus communis*) (Table 3). Characteristic of these plants was the absence of ferulic acid, present in all other mistletoes. It is also worth

Table 3. The content of phenolic acids in mistletoes hosted by *Malus domestica* L. and *Pyrus communis* L.

Comp. No.	Phenolic acids	*Malus domestica L.*			*Pyrus communis L.*		
		A	B	C	A	B	C
1.	trans–cinnamic	1.27	–	traces	0.32	–	0.15
2.	gallic	3.74	5.67	2.22	2.21	3.87	2.17
3.	p–hydroxybenzoic	–	–	–	–	–	–
4.	protocatechuic	–	–	1.04	–	–	0.79
5.	isochlorogenic	–	–	–	–	–	–
6.	chlorogenic	0.84	4.27	–	3.82	1.33	–
7.	caffeic	0.17	0.86	0.09	1.28	1.42	3.14
8.	gentisic	–	–	1.65	–	–	0.99
9.	syringic	5.62	–	0.47	6.97	–	1.62
10.	isoferulic	–	–	–	–	–	–
11.	rosmarinic	17.48	–	–	14.32	–	–
12.	p–coumaric	3.22	–	1.74	4.71	–	0.88
13.	m–coumaric	11.77	–	–	9.22	–	–
14.	veratric	4.47	–	–	2.76	–	–
15.	ferulic	–	–	–	–	–	–
16.	sinapic	traces	–	–	0.08	–	–
17.	vanillic	5.52	3.48	–	4.82	0.34	–
18.	digallic	–	–	–	–	–	–
19.	salicylic	–	–	–	–	–	–
20.	ellagic	–	–	–	–	–	–
21.	o–coumaric	–	–	–	–	–	–

For explanation: A,B,C see Table 1

Table 4. The content of phenolic acids in mistletoes hosted by *Populus nigra L.* and *Quercus robur L.*

Comp. No.	Phenolic acids	*Populus nigra L.*			*Quercus robur L.*		
		A	B	C	A	B	C
1.	trans–cinnamic	0.36	1.42	2.76	0.89	0.17	2.18
2.	gallic	11.87	10.64	5.21	11.42	8.53	9.42
3.	p–hydroxybenzoic	–	traces	–	traces	traces	–
4.	protocatechuic	3.42	–	1.22	5.58	–	2.44
5.	isochlorogenic	8.21	9.45	–	10.15	2.84	–
6.	chlorogenic	16.11	12.34	–	21.74	7.22	–
7.	caffeic	1.6	0.88	0.03	0.12	traces	–
8.	gentisic	3.22	7.14	traces	5.18	4.47	0.05
9.	syringic	traces	–	–	–	–	–
10.	isoferulic	–	–	–	–	–	0.42
11.	rosmarinic	–	–	–	2.11	–	–
12.	p–coumaric	2.33	–	0.12	4.12	0.34	–
13.	m–coumaric	–	–	–	traces	–	–
14.	veratric	–	–	–	–	–	–
15.	ferulic	0.22	3.72	2.61	0.09	0.58	1.14
16.	sinapic	–	–	–	–	–	–
17.	vanillic	–	–	–	–	–	–
18.	digallic	–	–	–	–	–	–
19.	salicylic	8.21	7.42	–	1.89	0.74	–
20.	ellagic	2.72	3.81	–	–	–	–
21.	o–coumaric	–	–	–	–	0.25	–

For explanation: A,B,C see Table 1

noting that m–coumaric acid, apart from these two mistletoes, was only present in *Viscum album* hosted by *Quercus robur*. Like in the plants hosted by *Acer plantanoides*, the analysed fractions did not contain salicylic acid, which was the main component of the set of phenolic acids in *Viscum album* hosted by *Sorbus aucuparia*. (Table 1).

In *Viscum album* hosted by *Populus nigra* 13 free and combined phenolic acids were identified. (Table 1). The main component of the analysed set of compounds was chlorogenic acid. (Table 4). The acid was present in the free form (fraction A) (16.11 mg%) and combined as glycoside (12.34 mg%). This is a significant quantity. In addition, isochlorogenic acid was identified, which was only present in plants hosted by *Acer plantanoides* and *Quercus robur* (Table 1). Like in the plant hosted by *Sorbus acuparia*, rosmarinic acid was not identified.

The mistletoe hosted by *Quercus robur* proved to be the richest in phenolic acids. HPLC analysis revealed 15 compounds present in this plant material. In this set, o–coumaric acid is only present combined as a glycoside and isoferulic acid as an ester. It is worth noting that isoferulic acid was only identified in the plant hosted by *Quercus robur*. (Table 1). This compound (0.42 mg%) seems to be characteristic of this plant only (Table 4). Other phenolic acids, i.e. rosmarinic, m–coumaric and digallic acids are present in free forms. The dominant element of the set is chlorogenic acid. (Table 4). The compound is present in exceptionally large quantities (21.74 mg% – fraction A and 7.22 mg% – fraction B). Also, oak–based mistletoe showed no presence of syringic acid, present in all other batches of the plant material.

Summing up, the HPLC analysis of mistletoes gathered from 6 different hosts proved that the plants are rich in phenolic acids, which could be related to the immunostimulating properties of this plant.

Research also indicated certain differences between the analysed mistletoes in terms of type and quantity of phenolic acids present. O–coumaric acid seems to be typical of mistletoe gathered from *Quercus robur*, digallic acid of the plant gathered from *Acer plantanoides* and vanillic acid for the plant gathered from *Malus domestica* and *Pyrus communis*. Worthy of notice are also the similarities between the two last batches of plant material in terms of phenolic acids contained.

The differences in the qualitative composition of phenolic acids discussed above may be helpful in analysing mistletoes gathered from different hosts. In case of plants hosted by *Quercus robur* it seems particularly significant that it is exceptionally rich in phenolic acids.

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