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DETERMINATION OF SOME POLYPHENOLIC COMPOUNDS IN BUDS OF *Alnus* AND *Corylus* SPECIES BY HPLC

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The presence of 19 polyphenolic compounds was simultaneously assessed through a HPLC method in the buds of three species: Alnus glutinosa, Alnus incana, *and* Corylus avellana. *The pattern of phenol carboxylic acids indicates that compounds like gentisic acid, sinapic acid, and the esters of tartaric acid (cichoric acid, caftaric acid) do not exist in buds, although they have been detected in mature organs. Buds only contain esters of quinic acid. Ferulic acid is a common compound in buds, being observed in all genus investigated. In the case of the flavonoid profile, young tissues of* Alnus sp. *and* Corylus avellana *only contain glycosides of quercetin. The preferred sugar for glycosylation is galactose, as the major flavonoid glycoside is in all cases hyperoside. The buds of* A. glutinosa *display the highest content in phenol carboxylic acids; this situation justifies their main prescription in the treatment of hepatic disorders.*

Key words: polyphenolic compounds, buds, *Alnus glutinosa*, *Alnus incana*, *Corylus avellana*, HPLC-DAD.

Polyphenolic compounds constitute one of the largest, most widespread and functionally important groups of secondary plant metabolites. Presently they are divided into several classes: phenol carboxylic acids, flavonoids, tannins, coumarins, anthranoids, and lignans [1]. In recent years, these substances, and flavonoids in particular, have attracted great interest due to their antioxidative capacity, which confers on them a valuable therapeutic potential in treating a large array of free-radical mediated diseases [2].

The numerous health benefits of polyphenols promoted a wide-scale investigation of their presence in plant products. However, in the case of buds (*gemmae*), pharmacognostic researches have only been undertaken to a small extent. Recent histochemical tests revealed the presence of three types of polyphenolic compounds in buds, differing in what their intracellular localization is concerned: granular polyphenols, vacuolar polyphenols, and drop-like polyphenols [3].

The present study was aimed at bringing new data on the polyphenolic profile of *Alnus* and *Corylus* buds, closely related from a phylogenic point of view. These buds are important gemmotherapeutic remedies, prescribed in hepatic, respiratory, circulatory, and inflammatory disorders [4]. However, data on their chemical composition are lacking as until the present, phytochemical studies have only been performed on barks, roots and root exudates, leaves, fruits, and seeds of *Alnus* sp. and *Corylus avellana* [5–10]. Beyond giving information on the composition of these buds, the present research is also expected to give clues regarding the ontogenic dynamics of polyphenols in the two mentioned genus, and to create the base for a scientific and rational therapeutic utilization of these buds. Our investigations employed, after adaptation, a previously developed HPLC method for the simultaneous determination of phenolic compounds [11, 12].

Nineteen polyphenolic compounds have been researched in the foliar buds of *Alnus glutinosa* (L.) Gaertn., *Alnus incana* (L.) Moench, and *Corylus avellana* L. The substances were: one hydroxybenzoic acid, seven cinnamic acid derivatives (free and esteric), four quercetin glycosides, and seven aglycones of flavonol and flavone type. The results are summarized in Table 1; the polyphenolic compounds are shown in order of their retention time. Quantitative determinations were performed using an external standard method.

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TABLE 1. The Content in Polyphenolic Compounds of Foliar Buds of *Alnus glutinosa*, *Alnus incana*, and *Corylus avellana* (mg/100 g fresh buds)

Polyphenols	*Alnus glutinosa*		*Alnus incana*		*Corylus avellana*	
	NH	H	NH	H	NH	H
Caffeic acid	-	-	-	-	-	0.11
Chlorogenic acid	-	-	-	-	0.38	0.30
p-Coumaric acid	-	1.42	-	0.53	-	-
Ferulic acid	-	0.50	-	0.26	0.13	0.41
Hyperoside	6.36	-	3.82	-	3.91	-
Isoquercitrin	-	-	1.56	-	1.56	-
Quercitrin	-	-	-	-	1.20	-
Quercetin	-	3.97	-	2.94	-	4.11

NH - nonhydrolyzed sample; H - hydrolyzed sample.
Not found: caftaric acid, gentisic acid, sinapic acid, cichoric acid, rutoside, myricetin, fisetin, patuletin, luteolin, kaempferol, apigenin.

TABLE 2. Retention Time of Polyphenolic Compounds, min

Peak nr.	Phenolic compound	t_R±SD	Peak nr.	Phenolic compound	t_R±SD
1	Caftaric acid	3.34±0.06	11	Rutoside	20.76±0.15
2	Gentisic acid	3.83±0.07	12	Myricetin	21.13±0.12
3	Caffeic acid	6.12±0.04	13	Fisetin	22.91±0.15
4	Chlorogenic acid	6.83±0.05	14	Quercitrin	23.64±0.13
5	p-Coumaric acid	9.48±0.08	15	Quercetin	27.55±0.15
6	Ferulic acid	12.80±0.10	16	Patuletin	29.41±0.12
7	Sinapic acid	15.00±0.10	17	Luteolin	29.64±0.19
8	Cichoric acid	15.96±0.13	18	Kaempferol	32.48±0.17
9	Hyperoside	19.32±0.12	19	Apigenin	39.45±0.15
10	Isoquercitrin	20.29±0.10			

t_R - retention time; SD - standard deviation.
Chromatographic conditions are described in the section “Experimental”.

The pattern of phenol carboxylic acids indicates the absence of gentisic acid and sinapic acid in buds, as well as that of the tartaric esters: cichoric and caftaric acids. These compounds do not appear in buds, although they have been observed in mature organs [10]. Ferulic acid could be found in all genus investigated; in *Alnus* sp. it exists only in esteric forms, whereas the buds of *Corylus avellana* contain this compound in both free and esteric form. *p*-Coumaric acid was detected after hydrolysis only in the buds of *Alnus glutinosa* and *A. incana*, the first species being 2.7 times richer in this compound. In contrast, caffeic acid could be found only in *Coryli gemmae*, where it exists natively as an ester with quinic acid, forming chlorogenic acid. From the esterified phenol carboxylic acids, only chlorogenic acid could be detected (in *C. avellana* buds).

In case of flavonoids, the flavonic aglycons apigenin and luteolin are absent in all samples. The flavonols, kaempferol, patuletin, myricetin, and fisetin, could not be detected, neither as free aglycons nor after hydrolysis. However, all samples contain quercetol in glycoside form: *A. glutinosa*, as hyperoside; *A. incana*, as hyperoside (mainly) and isoquercitrin; *C. avellana*, as hyperoside (mainly), isoquercitrin and quercitrin (in close quantities). The additional presence of isoquercitrin in *A. incana* buds represents a potential taxonomic marker that differentiates the two investigated *Alnus* species.

The profile of the phenolic compounds in buds shows some interesting differences in comparison with that of mature organs. Amaral et al. [10] detected in hazelnut leaves the following phenolic compounds: 3-caffeoylquinic acid, 5-caffeoylquinic

acid, caffeoyltartaric acid, *p*-coumaroyltartaric acid, myricetin 3-rhamnoside, quercetin 3-glycoside (isoquercitrin), quercetin 3-rhamnoside (quercitrin) and kaempferol 3-rhamnoside. In the bark (cortex) and roots of various *Alnus* sp., hyperoside, kaempferol and quercetin have been discovered [6, 7]. Unlike the mature organs, the buds of hazelnut and alder do not contain kaempferol or myricetin glycosides. A further difference refers to the type of glycosides: in hazel buds the major polyphenolic glycoside is hyperoside (not found in mature leaves), whereas in leaves, myricetin 3-rhamnoside and quercetin 3-rhamnoside are the major compounds [5].

These considerations suggest that in the early stages of development, the plant tissues of *Alnus* sp. and *Corylus avellana* only contain glycosides of quercetin. Later, quercetin is subjected to metabolic transformations, like the removal/adding of hydroxyl groups, diversifying the flavonoid patrimony. Also, the sugar moieties are diversified in mature organs. In buds, the preferred sugar for glycosylation is galactose, as the major flavonoid glycoside is in all cases hyperoside; later, the glycosilation preferentially occurs with rhamnose.

The buds of *A. glutinosa* prove to have, from all 3 species researched, the highest content in phenol carboxylic acids, due mainly to the presence of *p*-coumaric acid. This situation justifies their main indication in the treatment of hepatic disorders, as phenol carboxylic acids are known for their choleretic effect [1]. The hydroxycinnamic acids also possess anti-inflammatory activity, which, doubled by the antioxidative effect of flavonoids, is beneficial in a number of disorders, where reactive oxygen species intervene in the chain of pathogenic reactions. Due to their low content in active compounds, the buds of *Alnus incana* should preferably not be used.

Comparing the two *Alnus* species, *A. glutinosa* is devoid of isoquercitrin, but it contains higher amounts of both flavonoids and phenol carboxylic acids than *A. incana*; these aspects potentially represent taxonomic markers.

The buds of *A. glutinosa* prove to have, from all 3 species researched, the highest content of phenol carboxylic acids. This situation justifies their main indication in the treatment hepatic disorders. Buds of *Alnus incana* should preferably not be used, as their content of the active principles is low. The buds of *C. avellana* contain the largest amounts of the flavonoids investigated when compared to *Alnus* buds. This justifies their preferential employment in inflammatory diseases.

The pattern of phenol carboxylic acids indicates that compounds like gentisic acid, sinapic acid, and the esters of tartaric acid (cichoric acid, caftaric acid) do not exist in buds, although they have been detected in mature organs. Buds only contain esters of quinic acid. Ferulic acid is a common compound in buds, being observed in all genus investigated. In case of the flavonoid profile, young tissues of *Alnus* sp. and *Corylus avellana* only contain glycosides of quercetin. Later, quercetin is subjected to metabolic transformations, generating other aglycons; also, the sugar moieties are diversified in mature organs. In buds, the preferred sugar for glycosylation is galactose, as the major flavonoid glycoside is in all cases hyperoside.

EXPERIMENTAL

Apparatus and Chromatographic Conditions. The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser G1322A, quaternary gradient pump G1311A, and autosampler G 1313 A. For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18 100 × 3.0 mm i.d., 3.5 μm particle); the work temperature was 48°C. The detection of the compounds was performed at 330 nm using a G1315A diode array detector system. The chromatographic data were processed using ChemStation software from Agilent, USA.

The mobile phase was a binary gradient prepared from methanol and buffer solution. The buffer solution was prepared by dissolving potassium dihydrogen phosphate (40 mM) in water, and the pH was adjusted to 2.3 with 85% orthophosphoric acid. The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; isocratic elution followed for the next 3 minutes with 42% methanol. The flow rate was 1 mL min^{-1} and the injection volume was 10 μL. All solvents were filtered through 0.5 μm filters (Sartorius) and degassed through ultrasonication.

Identification and Quantitative Determinations of Polyphenols. The compounds were detected at 330 nm. Retention times were determined with a standard deviation ranging from 0.04 to 0.19 min (Table 2). The detection limits were calculated as the minimal concentration producing a reproductible peak with a signal-to-noise ratio grater than 3. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5–50 μg/mL range with good linearity (R2 > 0.999) for a five point plot were used to determine the concentration of polyphenolics in plant samples.

Accuracy was checked by spiking samples with a solution containing each phenolic compound in a concentration of 10 mg/mL. All compounds were identified in samples by both standard addition and comparison of their retention times, and UV spectra with those of standards in the same chromatographic conditions.

Plant Material. The foliar buds of *Alnus glutinosa* (L.) Gaertn., *Alnus incana* (L.) Moench, and *Corylus avellana* L. were gathered in March 2005 from the wild flora of the Cluj-Napoca district, Romania. The vegetal material was positively identified at the Department of Pharmaceutical Botany, Faculty of Pharmacy, "Victor Babes" University of Medicine and Pharmacy Timisoara, and voucher specimens were deposited in the Herbarium of this institution.

Samples of Preparation. Samples of 100 mg air-dried, powdered buds were placed in 10 mL centrifuge tubes and subjected to different extraction procedures.

A. For the extraction of phenolic compounds as glycosides, 2 mL methanol, 2 mL water, and 0.2 mL ascorbic acid solution (100 mg/mL) were added to the samples. The mixtures were heated at 80°C on a water bath for 30 min, subsequently ultrasonicated for 15 min, and finally heated for another 30 min at 80°C. During the heating, 1 mL methanol was added every 10 m in order to ensure a permanent presence of methanol in the extraction process. After the extraction, the mixtures were centrifuged at 4000 r/min and the remaining solids were extracted two more times with 5 mL water using the same procedure. The combined extracts were diluted with water in a 25 mL volumetric flask and filtered through a 0.45 μm filter before injection.

B. For the extraction of free aglycons, the samples of powdered buds were treated with 2 mL methanol, 2 mL 2 M hydrochloric acid (for hydrolysis of glycosides), and 0.2 mL ascorbic acid solution (100 mg/mL) (as antioxidant). The mixtures were then subjected to extraction as described above.

Chemicals. Methanol of HPLC analytical-grade, 85% orthophosphoric acid, potassium dihydrogen phosphate, and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Standards: Caffeic acid, chlorogenic acid, *p*-coumaric acid, kaempferol, apigenin, rutoside, quercetol, quercitrin, isoquercitrin, myricetol, fisetin, hyperoside from Sigma (Germany), ferulic acid, sinapic acid, gentisic acid, patuletin, luteolin from Roth (Germany); cichoric acid, caftaric acid from Dalton (USA). Methanolic stock solutions (100 g/mL) of the above standards were prepared and stored at 4°C, protected from daylight; before being used as working solutions, they were appropriately diluted with double distilled water.

REFERENCES

1. R. Hansel and O. Sticher, *Pharmakognosie. Phytopharmazie*, 7th ed., Springer Verlag, Berlin–Heidelberg–New York, 2004, p.735.
2. K. R. Narayana, M. S. Reddy, M. R. Chaluvadi, and D. R. Krishna, *Indian J. Pharmacol*., **33**, 2 (2001).
3. J. Bilkova, J. Albrechtova, and J. Opatrna, *J. Exp. Bot.*, **35**, 336, 1129 (1999).
4. F. Pitera, *Compendio di gemmoterapia clinica*, 3rd ed, Ferrari Editore, Genova, 2000, 12 pp.
5. D. DeMerlier, A. Chandelier, N. Debruxelles, M. Noldus, F. Laurent, E. Dufays, H. Claessens, and M. Cavelier, *J. Phytopathol*., **153**, 2, 99 (2005).
6. M. A. Kulagina, O. V. Radko, and A. G. Serbin, *Visnik Farmatsii*, **3**, 17 (2004).
7. M. Hughes, C. Donnelly, A. Crozier, and C. T. Wheeler, *Can. J. Bot*., **77**, 9, 1311 (1999).
8. Z. Jerzmanowska, Z. Kraczewska, and A. Wladyslawa, *Roczniki Chemii*, **45**, 6, 1115 (1971).
9. B. Pasich, Z. Kowalewski, and P. Grzybek, *Dissertationes Pharmaceuticae et Pharmacologicae*, **18**, 4, 385 (1966).
10. J. S. Amaral, F. Ferreres, P. B. Andrade, P. Valentao, C. Pinheiro, A. Santos, and R. Seabra, *Nat. Prod. Res*., **19**, 2, 157 (2005).
11. S. Leucuta, L. Vlase, S. Gocan, L. Radu, and C. Fodorea, *J. Liquid Chrom. Rel. Technol*., **28**, 3109 (2005).
12. C. S. Fodorea, L. Vlase, S. E. Leucuta, and M. Tamas, *Chem. Nat. Comp*., **41** (4), 400 (2005).