Applied Biochemistry and Microbiology, Vol. 41, No. 1, 2005, pp. 94–98. Translated from Prikladnaya Biokhimiya i Mikrobiologiya, Vol. 41, No. 1, 2005, pp. 107–112.
Original Russian Text Copyright © 2005 by Shul'ts, Bakhvalov, Martem'yanov, Petrova, Syromyatnikova, Shakirov, Tolstikov.

Effects of Natural and Artificial Defoliation on the Content and Composition of Extractive Substances in Birch Leaves

E. E. Shul'ts*, S. A. Bakhvalov, V. V. Martem'yanov**, T. N. Petrova*, I. N. Syromyatnikova**, M. M. Shakirov*, and G. A. Tolstikov***

**Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Division, Russian Academy of Sciences, Novosibirsk, 630090, Russia*
e-mail: schultz@nioch.nsc.ru
***Institute of Animal Systematics and Ecology, Siberian Division, Russian Academy of Sciences, Novosibirsk, 630091, Russia*
e-mail: bahvalov@online.sinor.ru

Received February 25, 2004

Abstract—Qualitative and quantitative compositions of extracts of birch (*Betula pendula* Roth.) leaves after natural and artificial defoliation were studied. Composition of the fraction of total lipids was determined. Overall, 11 fatty acids were identified. Differences between the fatty acid compositions of total lipids in the trees subjected to defoliation, consisting in the increase in quantities of short-chain saturated fatty acids and trienoic acids, were detected. Nine individual compounds—six flavones, two flavanones, and one flavanonol—were isolated from the flavonoid fraction by column chromatography. It was found that the total content of extractive substances in birch leaves as well as amounts of free sterols, triterpene compounds, and flavones decreased 1 year after artificial defoliation and 1 month after depredation of 75% of birch stands by gypsy moths. On the contrary, the contents of flavanones and flavanonol increased. The assay method proposed may be used for studying the compositions of plant extracts.

The changes inflicted on plants (within the same population) by herbivorous insects are among the vital aspects of population dynamics [1, 2] as well as of evolution [3] of the plant–insect–parasite system. Analysis of the literature data demonstrates that good quality of a forest stand and foliage as well as the content of protective substances in leaves are interconnected and allow the number of phyllophages and their parasites to be predicted with a high probability [2, 4, 5]. Defoliation of plants enhances their resistance to insects, resulting in a decrease in the viability of phyllophages and an increase in their sensitivity to pathogens [3, 4, 6–8]. In this process, secondary metabolites are thought important, as they provide active protection of plants from insects and under certain physiological conditions, may be preserved by plants and produced in larger quantities. The plant chemical compounds responsible for resistance to insects are manifold (terpenoids, various phenolic compounds, and condensed tannins) [1, 6–8], making development of general analytical methods for their quantitative determination rather difficult. However, study of the component composition of extractive substances as well as determination and isolation of the compounds produced by plants in response to defoliation stress is topical and important, in particular, for solving a number of ecological problems.

The goal of this work was to develop an efficient analytical method and use it for studying the composition and concentration dynamics of extractive substances in leaves upon defoliation of the European white birch (*Betula pendula* Roth.)—the main feeding tree for the major birch forest pest in Western Siberia, the gypsy moth (*Lymantria dispar* L.).

MATERIALS AND METHODS

Preparation of material. European white birch (*Betula pendula* Roth.) leaves were collected in natural birch woods during the mass reproduction of gypsy moths (Novosibirsk oblast, Russia). Samples **Xp1** were produced by extraction of the leaves collected 30 days after artificial defoliation with loss of 75% of the foliage; samples **Xp2**, by extraction of the leaves collected 1 year after artificial defoliation from the same trees. The extractive substances **Xp3** were obtained from the leaves of the trees with 75% of the foliage depredated during the previous 2 years. The leaves with normal foliage were used as a control (**Xc**). Extractive substances were isolated upon drying the leaves at room temperature.

Isolation of extractive substances. Air-dried ground leaves (40 g) were extracted with ether 3 × 150 ml) in the dark (20°C) for 2 days. The extracts were pooled, washed with water, and dried with magnesium sulfate;

Table 1. Total content of extractive substances and secondary metabolites in leaf samples (% of air-dried material weight)

Extract	Xp1	Xp2	Xp3	Xc
Extractive substances	6.44	5.02	4.27	5.85
Including: Extracts 1 and 2	1.82	1.52	1.10	2.11
Extract 3	2.42	2.61	2.54	1.79
Extract 4	0.96	0.64	0.25	1.43
Extract 5	1.24	0.25	0.38	0.52
Simple polyphenols and oxybenzoic acids	0.41*	0.34	0.39	0.40
Flavonoids of extract 3	2.12*	1.92	2.80*	2.32*
Flavonoids of extract 4	0.37	0.24	0.40	0.26
Flavonoids of extract 5	0.37	0.51	0.27	0.37

* Significant difference between control and damaged trees ($P < 0.05$).

the solvent was evaporated to dryness. The residue was dissolved in 10 ml of methanol under heating and then cooled. The precipitated wax fraction (extract 1) was separated and assayed. The methanol solution was supplemented with 0.5 g of KOH solution in 40 ml of methanol and boiled for 2 h. The hydrolysate was diluted with water (4 ml) and extracted with ether (4 × 100 ml; extract 2). Upon extraction with ether, the aqueous phase was neutralized and re-extracted with ether (extract 3). The plant material was placed into 50 ml of aqueous methanol, extracted at 20°C for 2 days, and evaporated to one half of the volume, in order to extract with 98% methyl-*tert*-butyl ether, followed by drying and evaporation (extract 4). The aqueous phase was supplemented with 1.5 ml of concentrated HCl, boiled for 30 min, cooled, neutralized with 10% solution of $NaHCO_3$, and extracted with 97% methylene chloride. The organic phase was dried with anhydrous magnesium sulfate and evaporated (extract 5). The yields of extractive substances from various fractions are listed in Table 1.

NMR spectra. ^{1}H NMR spectra of the total fractions and individual compounds were recorded in a Bruker AC 200 (Bruker, Germany) spectrometer (working frequency, 200.13 MHz) for 5% solutions of $CDCl_3$, $(CD_3)_2CO$, or CD_3OD.

Mass spectra were recorded using a Finnigan MAT-8200 (the Netherlands) mass spectrometer at an ionization potential of 70 eV and evaporator temperature of 270–300°C. The total fractions of lipids and methylation products were assayed by chromatography–mass spectrometry in a Hewlett-Packard 5890/II MSD (Hewlett, Germany) gas chromatograph equipped with a HP MSD 5971 quadrupole mass spectrometer as a detector. A 30-m HP-5MS quartz column with an inner diameter of 0.25 mm and a thickness of the stationary phase film of 0.25 μm with copolymer of 5% biphenyl–95% dimethylene siloxane was used. Chromato-mass spectra were recorded under the following conditions: the initial column temperature was 50°C; temperature was elevated at a rate of 4°C/min and then kept at 280°C for 15 min. The percentage compositions of fatty acids and methyl esters of fatty acids and alcohols were calculated according to the areas of gas chromatography peaks without correction coefficients.

Compositions of individual fractions were subjected to preliminary assessment according to ^{1}H NMR spectra of the total fractions. Upon hydrolysis and methylation, extract 1 was used for determination of fatty acids. Extract 2 contained a mixture of neutral lipids, terpene compounds, and fatty alcohols.

The main groups of phenolic compounds were found in total extracts 3–5. Extract 3 contained simple phenols, hydroxybenzoic acids, and free flavonoids. According to ^{1}H NMR data, extract 4 contained the major part of flavonoid glycosides. Components of extract 5 were mainly condensed polyphenols and anthocyanins.

Results of determination of the fatty acid compositions of acyl-containing fractions and fatty alcohols are listed in Tables 2 and 3.

The fractions of sterols and terpene compounds were isolated by column chromatography on silica gel. The contents of β-sitosterol and a derivative of betulafolienetetraol (triterpene 1) was determined according to chromatography–mass spectroscopy. The results are shown in Table 4. Total polyphenols of individual fractions were determined photometrically according to the reaction with 1% vanillin in concentrated HCl [9].

Total flavonoids were determined spectrophotometrically by measuring the optical density of the complex formed by flavonoid compounds with aluminum chlo-

Table 2. Composition of fatty acids in birch leaves

Sample	Fatty acids, %											
	14 : 0	16 : 0	16 : 2	18 : 0	18 : 2	18 : 3	20 : 0	22 : 0	23 : 0	24 : 0	26 : 0	Total
Xp1	0.54	3.68	0.73	0.62	0.79	3.20	0.56	0.42	0.29	0.78	0.23	11.78
Xp2	0.46	5.56	–	0.78	1.62	6.51	1.98	1.01	–	0.51	0.40	18.86
Xp3	2.35	12.55	2.58	0.17	0.12	2.14	0.94	2.20	–	0.44	0.28	23.71
Xc	0.19	2.88	0.10	0.44	0.73	1.57	2.33	1.94	0.33	0.97	0.34	11.85

Table 3. Composition of fatty alcohols in extracts of birch leaves

Sample	Fatty alcohols, %						
	16 : 0	18 : 0	20 : 0	20 : 1	22 : 0	24 : 0	Total
Xp1	2.73	0.36	0.42	3.40	2.00	0.52	9.42
Xp2	1.60	0.31	1.48	8.34	4.24	1.26	17.23
Xp3	1.84	0.22	1.17	7.32	1.07	0.96	12.56
Xc	1.80	0.08	0.62	3.13	2.24	1.08	8.95

Table 4. Contents (% in dry substance) of free sterols and triterpene compounds

Compound	Sample			
	Xc	**Xp1**	**Xp2**	**Xp3**
β-Sitosterol	0.29	0.14	0.17	0.16
Triterpene 1	0.05	0.08	0.10	0.09
Total content of sterols and triterpenes	0.46	0.38	0.36	0.35

ride [10]. Gallic acid was used as a standard for plotting calibration graphs for simple phenols, gallotannins, and hydroxybenzoic acids; quercetins, for total flavonoids [11, 12]. Individual flavonoids (figure, **I**–**IX**) were isolated by column chromatography of extract 3 on silica gel KSK (Meaton, Russia; 0–140 μm) using a chloroform–ethanol (100 : 1 to 5 : 1) elution system. Structures of the compounds were determined from spectral data of individual substances by comparing with the spectra of known flavonoids [13].

The data were processed statistically by conventional methods [14].

RESULTS AND DISCUSSION

As is evident from Table 1, the yield of extractive substances of white birch *B. pendula* leaves amounted to 4.27–6.44%. Note that the plant response to loss of green mass was accompanied by a decrease in the total yield of extractive substances in leaves 1 year after artificial defoliation and after depredation by gypsy moths. The yield of these substances decreased mainly due to a reduction in the amount of flavonoid glycosides and polyphenolic compounds in extracts 4 and 5.

The response of plants to loss of green mass was accompanied by an increase in the contents of fatty acids and fatty alcohols in the total lipid fractions. Overall, 11 acids were identified in the total lipid extract (Table 2). The most represented saturated acids in the extracts were myristic ($C_{14:0}$), palmitic ($C_{16:0}$), arachic ($C_{20:0}$), and behenic ($C_{22:0}$) acids. Unsaturated acids were mainly represented by two compounds—linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids. Palmitic acid was predominant in all the extracts. Note that the content of saturated acids increased in birch leaves 1 year after artificial defoliation and 30 days after depredation by gypsy moths. In addition, it is evident from Table 2 that fatty acids are desaturated (new double bonds are formed) during development of new leaves after artificial defoliation. The leaves of plants in the case of natural defoliation display a higher content of short-chain saturated fatty acids (14 : 0 and 16 : 0) and trienoic acids compared with the control. Presumably, biochemical adaptation during depredation is regulated with the help of synthesis of polyunsaturated acids and the formation of fatty acids with short carbon chains. It is noteworthy that the content of saturated long-chain acids ($C_{20:0}$ and $C_{22:0}$) increased in the leaves of control group trees. Interestingly, linolenic acid is predominant in the total lipid extract during normal development of birch leaves from buds, while the ratio of unsaturated to saturated fatty acids ranges from 2 : 1 to 3 : 1 [15].

As previously mentioned, the fatty alcohol content also increased in the lipid extract of leaves from trees subjected to defoliation (Table 3). The major component of the mixture of fatty alcohols was diterpene phytol, a component of the molecules of chlorophyll and tocopherols. Interestingly, the highest content of phytol was found in extracts of leaves 1 year after natural and artificial defoliations and 30 days after depredation by insects.

Sterols and triterpene compounds, whose content amounted to 0.35–0.46% (Table 4), were isolated by column chromatography of extract 2. The major component of the sterol fraction in all leaf samples assayed was β-sitosterol. Its content decreased ($P < 0.05$) in the extracts of leaves from plants after defoliation. The major component of triterpene compounds was triterpene 1. This compound, which is a derivative of betulafolienetetraol, carries a substituted tetrahydrofuran cycle in the side chain; the mass spectrum contains peaks with *m/e* 143 (100%), which corresponds to the fragment ($C_8H_{15}O_2$), and *m/e* 59, corresponding to $(CH_3)_2C^+$–OH [16–18].

Much recent attention has been focused on the role of phenolic compounds when studying the plant–insect–parasite system [4, 7, 8, 19, 20]. We determined the quantitative contents of phenolic compounds in extracts 3–5 (Table 1), studied compositions of the fractions by column chromatography, isolated several individual compounds (figure), and determined the ratio of the main compounds according to the characteristic chemical shifts of protons in ^{1}H NMR spectra. The data listed in Table 1 demonstrate that the extracts of plants subjected to natural defoliation displayed the highest content of flavonoids.

Free *p*-methoxybenzoic, *trans*-*p*-coumaric, 3,4-dihydroxy-, and 3,5- dihydroxybenzoic acids were isolated from extract 3 of a control sample **Xc**. Characteristically, these acids were found in trace quantities in samples of the leaves after artificial and natural (browsing by gypsy moth) defoliations.

Table 5. Contents of flavonoids (I–IX) in extract 3

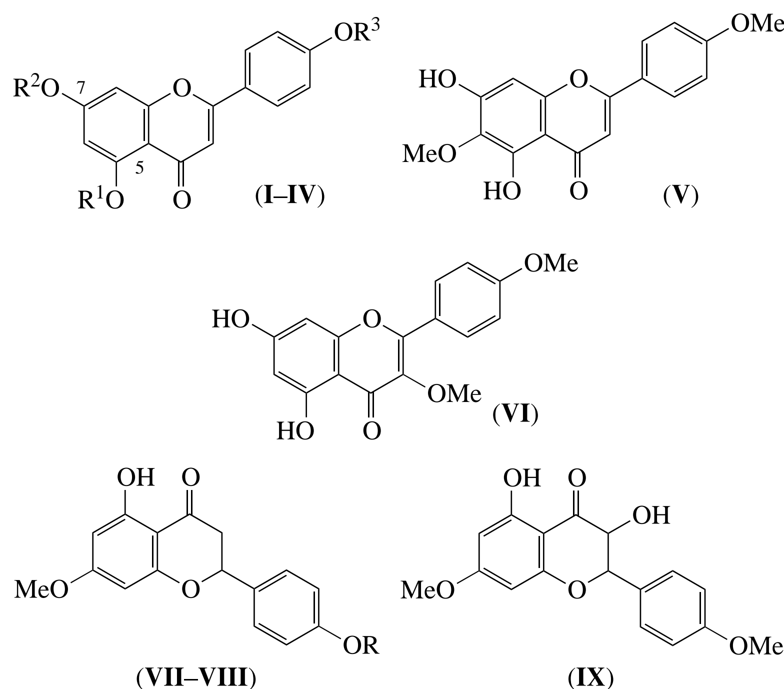
Sample	Content, % total flavonoids in Extract 3								
	I	II	III	IV	V	VI	VII	VIII	IX
Xp1	20.4*	10.4*	8.3*	7.5*	4.2	8.4*	6.8*	8.3	9.1
Xp2	15.4*	8.1*	5.1	4.5	8.8*	9.7*	14.4*	15.3*	10.1*
Xp3	10.5*	7.4*	6.7*	5.1*	11.1*	8.0*	16.4*	17.5*	11.1*
Xc	26.6	11.1	10.5	9.7	4.0	5.9	5.8	6.5	6.3

* Significant difference between control and damaged trees ($P < 0.05$ and 0.01).

The content of total flavonoids in birch leaf extracts amounted to 2.67–3.47%. Along with the quantitative determination of phenolic compounds, we studied the qualitative compositions of the fractions of extract 3. Column chromatography of the extracts of samples **Xp2**, **Xp3**, and **Xc** allowed six individual flavones (**I**–**VI**), two flavanones (**VII** and **VIII**), and one flavanonol (**IX**) to be isolated. When considering the structure of individual compounds (Table 5, figure), note that the main flavones and flavanones contain 5-hydroxy-7-methoxy- groups in the core (~30–40%). Of interest is the detection and isolation of 3-methoxyflavone (**VI**) and 3-hydroxyflavanonol (IX) in the extracts. Note that, according to [20], polymethoxyflavones belong to compounds rendering plants resistant to pathogenic fungi (e.g., *Penicillium digitatum* and various *Geotrichum* species).

The ratios of the main flavonoids in extracts differed considerably. Characteristically, the samples **Xp2** and **Xp3** displayed increased contents of flavanones (**VII**) and (**VIII**)—to 30–34% of the total extractive substances vs. 12% in the control. Note that defoliation also increased the concentration of flavanonol (**IX**). Presumably, biosynthesis of other types of aryl-benzo[d]pyrans for precursors is more active in the plants subjected to defoliation [21, 22]. The content of main flavanones (**I**)–(**III**) in the plants subjected to defoliation declined from 60 to 20%.

Further extraction of the samples with aqueous methanol allowed the glycoside derivatives of flavonoids to be concentrated. Analysis of NMR spectra of total fractions of extract 4 demonstrates that in all the cases, quercetin (3,5,7,3'4'-pentahydroxyflavone [13]) was the main aglycone of the flavonoid complex.



Structure of the phenolic compounds isolated: (**I**) $R^1 = R^3 = H$, $R^2 = Me$; (**II**) $R^1 = H$, $R^2 = R^3 = Me$; (**III**) $R^1 = R^2 = H$, $R^3 = Me$; (**IV**) $R^1 = R^2 = R^3 = Me$; (**VII**) R = H; and (**VIII**) R = Me.

Extracts 5 (Table 1) contained the bulk of tannins and anthocyanins. The total content determined spectrophotometrically is shown in Table 5. The ratio of these compounds to the total content of phenolic components varied in the range of 7–17%. Interestingly, the content of these compounds was considerably higher in leaves of the plants 1 year after artificial defoliation ($P < 0.05$) and the damage caused by gypsy moth ($P < 0.01$) compared with the control. These results are of certain interest in connection with the data reported in [2, 19] on the contents of anthocyanins, proanthocyanidins, and gallotannins in foliage of the birch trees resistant to insect damage.

Thus, a method for analysis of the extractive substances contained in plant leaves is proposed. The method allows composition of the components and their dynamics under natural or artificial defoliation stress to be evaluated. During defoliation, a decrease in the total content of extractive substances is observed due to a decline in the contents of sterols, triterpene compounds, and polar polyphenolic substances. Defoliation of trees is accompanied by an increase in the contents of short-chain and unsaturated fatty acids and a decrease in the flavone fraction in the total lipid extract; however, flavanones, flavanonol, and glycoside-containing flavones are concurrently accumulated.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project nos. 01-04-48965 and 04-04-48547).

REFERENCES

1. Louda, S.M. and Collinge, S.K., *Ecology*, 1992, vol. 73, no. 1, pp. 153–169.
2. Thompson, J.N., *Ann. Rev. Ecol. System.*, 1988, vol. 19, no. 1, pp. 65–87.
3. Ruohomaki, K., Chapin, III F.S., Haukioja, E., Neuvonen, S., and Suomela, J., *Ecology*, 1996, vol. 77, no. 8, pp. 2302–2311.
4. Haukioja, E., *Ann. Rev. Entomol.*, 1991, vol. 36, pp. 25 – 42.
5. Hanhimaki, S., *Oecologia*, 1989, vol. 81, no. 3, pp. 242–248.
6. Zvereva, E.L., Kozlov, M.V., Niemela, P., and Haukioja, E., *Oecologia*, 1997, vol. 109, no. 3, pp. 368–373.
7. Dillon, R.J. and Charnley, A.K., *J. Invertebrate Pathol.*, 1995, vol. 66, no. 1, pp. 72–75.
8. Muzatova, O.V., *Vestn. Nats. Akad. Nauk Belarusi*, 2000, no. 4, pp. 93–96.
9. Lukovnikova, G.A. and Yarosh, N.P., in *Metody biokhimicheskogo issledovaniya rastenii* (Methods of Biochemical Studies of Plants), Leningrad: Kolos, 1972, pp. 87–128.
10. Safronov, V.V., Sakanyan, E.I., and Lesiovskaya, E.E., *Rastit. Resursy*, 2000, vol. 36, no. 3, pp. 128–130.
11. Gins, V.K., Kolesnikov, M.P., Kononkov, P.F., Trishin, M.E., and Gins, M.S., *Prikl. Biokhim. Mikrobiol.*, 2000, vol. 36, no. 3, pp. 344–353.
12. Forsyth, W.G.C., *Biochem. J.*, 1955, vol. 60, no. 1, pp. 108–111.
13. Malikov, V.M. and Yuldashev, M.P., *Khim. Prir. Soedin.*, 2002, no. 4, pp. 299–324.
14. Lakin, G.F., *Biometriya* (Biometry), Moscow: Vysshaya shkola, 1974.
15. Vetchinnikova, L.V., Shulyakovskaya, T.A., and Kanyuchkova, G.K., *Rastit. Resursy*, 2000, vol. 36, no. 3, pp. 85–92.
16. Fischer, G. and Seiler, N., *Liebigs Ann. Chem.,* 1961, vol. 644, no. 1, pp. 146–171.
17. Nagai, M., Tanaka, N., and Ichikawa, S., *Chem. Pharm. Bull.*, 1973, vol. 21, no. 10, pp. 2061–2065.
18. Uvarova, N.I., Malinovskaya, G.V., El'kin, Yu.N., Isakov, V.V., Dzidenko, A.K., and Elyakov, G.B., *Khim. Prir. Soedin.*, 1976, no. 5, pp. 757–762.
19. Ossipova, S., Ossipov, V., Haukioja, E., Loponen, J., and Pihlaja, K., *Phytochem. Anal.*, 2001, vol. 12, no. 2, pp. 128–133.
20. Zaprometov, M.N., *Fenol'nye soedineniya* (Phenolic Compounds), Moscow: Nauka, 1993.
21. Goodwin, T.W. and Merser, E.I., *Introduction to Plant Biochemistry*, Oxford: Pergamon, 1983, vol. 2.
22. Springob, K., Nakajima, J.-C., Yamazaki, M., and Saito, K., *Nat. Prod. Rep.*, 2003, vol. 20, no. 3, pp. 288–303.