Phytochemical Studies of *Rhododendron Adamsii* Rehder. Quantitative Content of Fatty Acids in Leaves and Stems

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Abstract

The qualitative and quantitative content of fatty acids in leaves and stems of *Rhododendron adamsii* Rehd. has been for the first time studied using gas chromatography and gas chromatography–mass spectrometry. It was shown that the use of preliminary hydrodistillation of plant raw material under investigation allows to remove the compounds interfering the identification and quantitative determination of fatty acids content. There are both non-branched and isostructured fatty acids including polyunsaturated ones containing in plants, with the number of carbon atoms from 12 up to 30 (including odd numbers). Acids with cyclopropane fragment were revealed too, which allows to make the assumption concerning the features of metabolism in this plant. Total content of the acids in leaves is 6.2 mass %, in stems it is amounting to 2.4 mass %.

INTRODUCTION

Some plants of the *Rhododendron* genus, the members of the Heath (Ericaceae) family are long since used in folk medicine of various countries [1]. *Rhododendron adamsii* Rehder is used as a stimulant and tonic by the population of Buryatia, Mongolia and China. Decoctions and tinctures of it are used for colds and cardiac diseases; as a diuretic agent for cardiac edema [2], as well as an adaptogen [3]. A comprehensive phytochemical study of such plants is important for the understanding of metabolic processes occurring in the plants, as well as for the searching novel natural compounds those exhibit biological activity.

The purpose of the present work was to study the content of fatty acids in leaves and stems of *Rhododendron adamsii* Rehder.

EXPERIMENTAL

The plant raw material for chemical research was collected within a natural habitat and dried up to an air-dry condition. The raw material was harvested in July and August of 2005 in Tunkinskaya valley near Arshan settlement (Buryatia) and was stored in a dry dark place until carrying out the experiments. The distillation of volatile components was carried out in November 2005 using a hydrodistillation method according well-known technique [4] then raw material was repeatedly dried up. Lipid extracts were obtained in November and December 2006.

The extraction of lipid components was carried out according the technique described in the paper [5]. The plant raw material (8.20 g leaves, 20.19 g stems) was iteratively extracted with hexane (three times in 100 mL) and then with diethyl ether (three times in 100 mL) using agitation with a magnetic stirrer at room temperature. The duration of the extraction procedure with each portion of both the solvents was about 8–10 h. The hexane and ethereal extracts were joined together and then the solvents were removed in vacuum. The total weights of the extracts of leaves and stems amounted to 1.71 g and 2.08 g, respectively. The data on the amount of the obtained extracts, the weight and content of fatty acids (FA) are presented in Table 1.

The fatty acids were determined as methyl esters. In order to determine the content of free acids about 1 g of the obtained total lipid

TABLE 1

Quantitative content of fatty acids (FA) in *Rhododendron adamsii* Rehd. leaves and stems

Parameter	Leaves		Stems	
	Free acids	Total content	Free acids	Total content
Raw material mass, g	9.97	9.97	20.19	20.19
Total lipid extract mass, g	2.82	2.82	2.08	2.08
Mass of extract for hydrolysis, g	–	0.71	–	1.00
"Acidic" part mass after neutralization, g	–	0.27	–	0.34
Total content of FA methyl esters in a sample (GLC data), mass %	12.6	57.7	11.9	68.3
Mass of acids scaled to lipid extract, g	0.36	0.62	0.25	0.48
FA content scaled to raw material, %	3.6	6.2	1.2	2.4

extract was dissolved in 5–10 mL of diethyl ether, a catalytic methanol quantity (1-2 drops) [6] were added and then a fresh diazomethane solution [7] in diethyl ether was added until gas evolution stopped. The reacting stuff was matured during 30–40 min and then the solvent was removed in vacuum. The methylated extract was analyzed using GC and GC/MS tecniques.

In order to determine the total content of acids, including those existing in the plant in esterified state, an alkaline hydrolysis of total lipid extracts was carried out. An aliquot (10–15 mL) of 1 M NaOH solution in 80 % methanol was added to ca. 0.5–1.0 g of total extract (precise weighing) and then thermostatted at 50 °C during 1 h. The hydrolyzate obtained was cooled down to the room temperature, diluted with water up to 100 mL and then was extracted with diethyl ether in the portions of 20–25 mL until the organic phase became colorless. Ethereal extracts were put aside, and aqueous layer was neutralized with hydrochloric acid up to pH 3–4 and then it was repeatedly extracted with diethyl ether 3–5 times running. The ethereal extracts were joined together, washed out up to neutral reaction of washing water and then the solvent was removed in vacuo. The procedure of methylation of the sample obtained was similar to the described above.

The quantitative gas chromatographic analysis of the methylated extracts was carried out using an Agilent 6890 chromatograph with a flame ionization detector and an Agilent G1701AA Chemstation data processing system. There was an HP-5 quartz capillary column (5 % diphenylsiloxane and 95 % dimethylsiloxane copolymer) used, 30 m in length and 0.32 mm internal diameter; the thickness of the film of stationary phase was 0.25 μm. Helium was used as a carrier gas, with flow rate of 2 mL/min. For the increase in sensitivity and the improvement of reproducibility a splitless mode of injection was used. Both the injector and detector temperature values were 280 °C; the column temperature program mode was: holding 2 min at 50 °C, then heating at 10 °C/min up to 280 °C and isothermal holding at this temperature during 20 min The FA composition was calculated using the method of internal normalization, the content of individual acids was presented as a percentage of total mass of all the substances.

The gas chromatography–mass spectrometry analysis was carried out using an HP-6890 gas chromatograph with HP 5972 mass selec-

tive detector. There was an HP-5 MS quartz capillary column (5 % diphenylsiloxane and 95 % dimethylsiloxane copolymer) used, 30 m in length and 0.32 mm internal diameter; the thickness of the stationary phase film was 0.25 μm. Helium was used as a carrier gas, with flow rate of 0.8 mL/min, a splitless mode of injection was used both the injector and detector interface temperature values were 280 °C; the column temperature program mode was similar to the described above. The identification of compounds was carried out through the comparison of retention time values times of some known samples and the comparison of full experimental mass spectra with the mass spectrometric data from the NIST 02 MS database (175 000 compounds) included in the Agilent G1701AA Chemstation system.

RESULTS AND DISCUSSION

It should be noted that the analysis of phytogenous lipids is connected with some complications. In particular, in the studies of fatty acids from leaves and stems of *Rh. adamsii* by the GC and GC/MS techniques it was revealed, that the presence of a lot of volatile terpene components in the plant [8] causes a chromatogram to be complicated. This interferes with the identification and the determination of quantitative FA content (as methyl esters), in particular if the content of any component does not exceed 1 %. Thus it is impossible to determine all the variety of FA contained in the plant.

We have revealed that after plant raw material hydrodistillation, *i.e.* after the removal an ethereal oil from it, the composition of total lipid extract is considerably simplified, which allows not only identifying the compounds using mass-spectroscopic data, but also estimating their trace amounts.

According to the GC and GC/MS data, there are almost all the all acids with carbon atoms number from 12 up to 30 (Table 2) including odd numbers in *Rh. adamsii* leaves observed in the free state, and their mass content with respect to the weight of leaves is 3.6 %. The maximal relative content is characteristic for behenic acid (C22, 1.9 %). The content of the other acids varies from 0.1 up to 1.0 %; some acids (C13-*iso*, C15-*iso*, *etc.*) are present in trace amounts. Almost all free saturated acids with the number of carbon atoms from 12 up to 21 are present in leaves both as non-branched, and as isostructured compounds; the mass ratio w(*iso*)/w(non-branched) varies from 0.2 up to 2.6, *i.e.* there is no prevailing isomeric forms of the acids in leaves. The content of unsaturated acids in *Rh. adamsii* leaves is insignificant amounting to about 15 % of total free acids content.

In order to determine the total acid content we carried out alkaline hydrolysis of the obtained total extracts. Free acids and all the acids containing in the plant as esters were extracted into an alkaline solution.

The total content of the acids in *Rh. adamsii* leaves amounts to 6.2 % (see Table 1). About a half of the amount is presented by the group of C18 saturated and unsaturated acids; a maximal relative content among them is characteristic for linolenic acid (C18 : 3, 12.6 %). There are also palmitic acid (C16 : 0, the content is 9.2 rel. %) and linoleic acid (C18 : 2, the content is 8.2 rel. %) abundant in leaves, which amounts to more than 85 % of the mass of all the acids together with the other acids whose relative content exceeding 1 %.

The comparison of the fatty acid composition of hydrolized and non-hydrolized extracts of leaves demonstrates that all the acids are present in the free state in the plant in either amount. All the major acids are contained in the plant mainly in the state of esters, the ratio between forms free and ester state varying.

Just as in leaves, C18 acids form a great deal of total FA content in stems (see Table 2). The difference consists in the fact that linolic acid prevails, its content being 2.6 times higher compared to linolenic acid. The two acids in total amount to almost 70 % of the mass of all the acids contained in *Rh. adamsii* stems. One can observe an increased relative content of C16, C18 : 0, C20, C22 and C24 acids, the content of the others does not exceed 1 %. Just as in leaves, as well, the (*iso*-)/(non-branched) ratio in stems can be both more and less than 1, the etherisation degree (ester/free acid ratio) varies depending on the acid.

The use of mass spectrometric detection has allowed us to find out that there are two acids in *Rh. adamsii* leaves and stems with a cyclo-

TABLE 2

Retention time (min) and the relative content (mass %) of FA methyl esters in the extracts of *Rhododendron adamsii* Rehd. leaves and stems according to GLC data

Retention time, min	Acid	Leaves		Stems	
		Free acids	Total content	Free acids	Total content
13.80	C12-*iso*	0.2	0.2	0.2	0.3
14.23	C12	0.2	0.6	0.2	0.3
14.50	Nonanedioic acid	0.3	0.1	0.3	0.2
14.95	C13-*iso*	<0.1	0.6	<0.1	0.3
16.05	C14-*iso*	<0.1	0.2	<0.1	0.3
16.44	C14	0.1	1.3	0.1	0.4
17.10	C15-*iso*	<0.1	0.5	–	<0.1
17.47	C15	0.3	0.1	0.1	0.1
18.26	C16 : 1 (7)	0.1	0.8	<0.1	0.7
18.47	C16	0.5	9.2	1.1	5.9
19.06	C17-*iso*	0.1	0.8	–	0.1
19.18	C17(*cis*-cyclopropane-9,10-hexadecanoic acid)	0.1	0.2	–	0.2
19.40	C17	<0.1	0.3	<0.1	0.2
19.99	C18-*iso*	0.2	1.3	0.1	–
20.04	C18 : 2 (9,12)	0.5	8.2	2.4	31.0
20.11*	C18 : 3 (9,12,15)	0.9	12.6	1.3	15.1
20.11*	C18 : 1 (9)	Traces	1.9	0.5	3.9
20.30	C18	0.4	2.8	0.5	1.5
20.86	C19-*iso*	0.4	3.2	<0.1	1.0
21.00	C19(*cis*-cyclopropane-9,10-octadecanoic acid)	–	0.5	–	0.1
21.17	C19	0.4	0.4	0.2	0.1
21.80	C20 : 1 (11)	0.3	0.6	–	0.6
22.00	C20	0.3	2.5	0.6	2.0
22.50	C21-*iso*	0.2	0.7	<0.1	0.4
22.80	C21	0.3	0.3	0.1	0.3
23.39	C22 : 1 (13)	0.3	0.2	–	0.2
23.57	C22	1.9	3.1	0.8	1.3
24.31	C23	1.2	0.6	0.8	0.2
25.03	C24	1.0	3.1	1.4	1.2
25.96	C25	1.3	0.2	–	–
26.67	C26	0.4	0.5	1.2	0.4
28.49	C28	0.8	<0.1	–	–
29.87	C30	<0.1	<0.1	–	–
	Total	12.6	57.7	11.9	68.3

Note. C=C double bond position is indicated in parentheses.

*Oleic and linolenic acids were revealed in the same gas chromatographic peak. The total content of the acids was determined by GLC, and the molar ratio was estimated basing on mass spectrometric data.

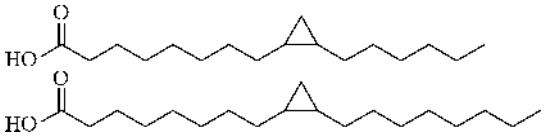

Fig. 1. Fatty acids with cyclopropane fragment.

propane fragment such as *cis*-cyclopropane-9,10-hexadecanoic acid and as *cis*-cyclopropane-9,10-octadecanoic acid (Fig. 1). The relative content of the acids amounts to 0.2 and 0.5 % with respect to the mass of the lipid extract of leaves as well as 0.2 and 0.1 % against the mass of the lipid extract of stems, respectively. The biosynthesis of such acids occurs through a methylene group transfer from S-adenosylmethionine to the double bond of monoenoic acids [9]. In general, such the acids are found out in bacteria (such as *Leuconostos oenos* [10], *Lactobacillus plantarum* [11], *etc.*), being rarely observed in plants (*Sterculia foetida* [9], *Litchi chinensis* [12], *etc.*). For the representatives of the *Rhododendron* genus, the data concerning the presence of such the acids are presented for the first time. In addition, nonanedioic acid was revealed, as well as hydroxy and methoxy derivatives of cinnamic acid were found out among aromatic acids. They are contained mainly in the state of esters, and their content does not exceed 1 % of the total mass of all the acids.

CONCLUSION

The leaves and stems of *Rh. adamsii* contain saturated and polyunsaturated C12–C30 fatty acids including "odd numbered" ones. Among free acids the maximal relative content is characteristic for behenic acid (C22, 1.9 %) and for linoleic acid (C18 : 2, 2.4 %) in stems. The overall content of fatty acids amounts to 6.2 and 2.4 % against the mass of leaves and

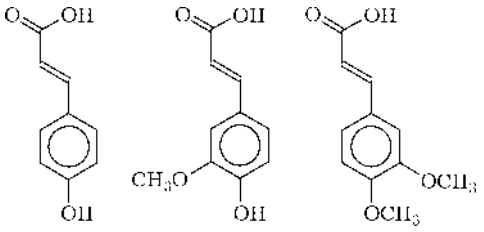

Fig. 2. Cinnamic acids of *Rhododendron adamsii* Rehd.

stems, respectively. The maximal content in leaves is characteristic for linolenic acid (C18 : 3, 12.6 %), in stems it is characteristic for linoleic acid (C18 : 2, 31.0 %).

Acids with cyclopropane fragment have been found out in a representative of *Rhododendron* genus for the first time. The relative content of such acids ranges from 0.1 to 0.5 % with respect to the mass of all the fatty acids in a plant.

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