Phytochemical Studies of *Rhododendron adamsii* Rehder. Quantitative Determination of Ursolic and Oleanolic Acids in Some Representatives of Ericaceae Family

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Abstract

Using HPLC, GC and GC/MS techniques, the quantitative total content and mass ratio value for ursolic and oleanolic acids have been determined for *Rhododendron adamsii* Rehd. leaves and stems as well as for *Oxycoccus palustris* (cranberry) and *Vaccinium praestans* Lamb. (krasniqi) berries press cake.

INTRODUCTION

The studies of composition and content of triterpene acids (TTA) in plant raw material is of important value not only from scientific point (phytochemical research), but also in the view of practice such as quality monitoring of the raw material, where the TTA are taken from. Among the TTA contained in a plant (ursolic, oleanolic, pomolic acids, *etc.*), ursolic acid mainly prevails (Fig. 1, *a*), oleanolic acid (isomeric with respect to ursolic acid (see Fig. 1, *b*) occurs too, and pomolic acid is more rarely observed.

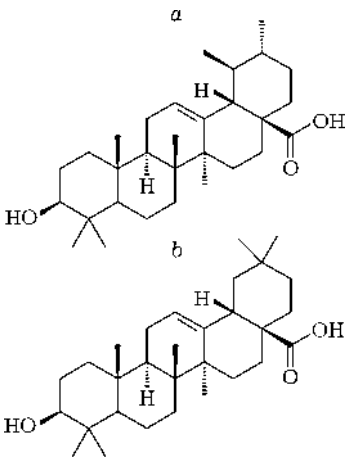

Fig 1. Ursolic *(a)* and oleanolic *(b)* acids.

Ursolic (UA) and oleanolic (OA) acids belong to triterpene acids of amirine series, are frequently observed in various plants being characteristic for Rosaceae and Ericaceae families [1]. Ursolic acid exhibits a great variety of bioactivities such as anticancer [2], antimutagene, antiviral, cytotoxic [3], as well as anti-inflammatory, anti-hyperlipidemic and anticancer promoting effects [4, 5]. Thereupon the investigations connected with searching for plant sources of UA as well as with quantitative determination of UK in these plants are of current importance.

The aim of the present work consisted in the development of a method that allows to determine UA and OA quantitative content in plant raw material, as well as in the approving the method for *Rhododendron adamsii* leaves and stems (as a continuation of our work [6] on *Rh. Adamsii* phytochemical studies) and for two other representatives of Ericaceae family such as *Oxycoccus palustris* (cranberry) and *Vaccinium praestans* Lamb. (krasniqi).

EXPERIMENTAL

Plant raw material and isolation procedure

The plant raw material for chemical research was collected within the natural habitat of each

species. *Rhododendron adamsii* Rehd. leaves and stems was harvested in July and August of 2005 in Tunkinskaya valley near Arshan settlement. (Buryatia), dried up to an air-dry condition and then stored in a dry dark place until carrying out the experiments. The raw material was subjected to a hydrodistillation procedure according well-known technique [7] and then it was repeatedly dried up.

The press cake of cranberry (*Oxycoccus palustris*) was obtained from the berries gathered in September and October 2004 in northern areas of the Novosibirsk Region under the conditions of industrial gathering. The presscake obtained was dried up to an air-dry condition and stored in a dry dark place. *Vaccinium praestans* Lamb. berries were gathered in August, 2006 in the area of Makarov city (Sakhalin Island) and stored in frozen state at −20 °C. Immediately before the experiments a presscake was made of them dried up to an air-dry condition.

The extraction of UA from plant raw material was carried out by benzene using a Soxhlet extractor during 2−3 days, the benzene extracts were filtered and then the solvent was removed in vacuum. An aliquot of methanol (1 mL) was added to ca. 20−30 mg of the extract obtained (precise weighing), the suspension was stirred up during 30−40 min using a shaker and centrifuged for 5−6 min. A supernatant was analyzed using HPLC technique. The efficiency of UA and OA extraction from the sample under analysis was checked using a repeated extraction of the obtained deposit by MeOH with further HPLC analysis.

Chromatographic analysis

The quantitative UA and OA content was determined using HPLC technique by means of Milichrom A-02 chromatograph (the Eco-Nova Institute of Chromatography, Novosibirsk). ProntoSil-120-5-C18-AQ sorbent was used as a stationary phase; a gradient from 85 % MeOH aqueous solution, with the addition of trifluoroacetic acid (0.1 % in volume), up to 100 % MeOH being used as the eluent. The time of analysis amounted to 20 min, the detection was carried out in a multiwavelength mode, the wavelength of 210 nm was chosen as a reference. The mass extinction values for UA and for UA methyl ester (98 % purity + according to GC) at the wavelength of 210 nm were 9.3 and 5.6 opt. units/mg, respectively.

In order to determine the quantitative ratio UA/OA, preliminary methylated extracts were analyzed using GC technique with a flame-ionization detector, the identification of compounds was carried out by means of GC/MS method. The quantitative UA and OA ratio values were determined according to ratio values for the areas of corresponding peaks of the chromatograms obtained.

Methyl esters were obtained through the treatment of total extracts by diazomethane solution; for that about 1 g of the total extract obtained was dissolved in 5−10 mL of diethyl ether, a catalytic methanol quantity (1−2 drops) [8] was added and then a fresh diazomethane solution [7] in diethyl ether was added until gas evolution stopped The reacting mixture was aged during 30−40 min and then the solvent was removed in vacuum.

The gas chromatography 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. An HP-5 quartz capillary column (5 % diphenylsiloxane and 95 % dimethylsiloxane copolymer) was used, 30 m in length and 0.32 mm internal diameter; the thickness of the stationary phase film amounted to 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 gas chromatography−mass spectrometry analysis was carried out using an HP-6890 gas chromatograph with an HP 5972 mass selective detector. There was an HP-5 MS quartz capillary column (5 % diphenylsiloxane and 95 % dimethylsiloxane copolymer) used, 30 m in length and 0.25 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 using comparison of full experimental mass spectra with the mass spectrometric data from the NIST 02 MS database (175000 compounds) included in the Agilent G1701AA Chemstation data processing system.

RESULTS AND DISCUSSION

There are several pathways in the literature described concerning the solution of the problem of UA determination in plant raw material. For example, UA derivatives are identified by means of HPLC technique after *p*-toluidine derivation [10], quantitative UA determination is carried out using HPTLC and HPLC assays in *Verbena officinalis* [11] and in *Potentilla discolor* Bge. [12], the presence of UA is verified by HPLC-MS method [13] and after silylation it is proved using GLC method [14]. A non-chromatographic tandem MS-MS method is described for UA determination in the some plants of *Staphylea* genus [15]. For a series of medicinal herbs growing in China, TTA were determined by means of capillary electrophoresis [16]. At the same time it should be taken into account that the quantitative determination of UA is complicated due to the presence of a structurally close isomer such as OA, as well as of other TTA and other masking compounds. The chromatographic separation of TTA from each other requires using the columns of high separation efficiency being possible only under GC conditions.

We believe that an appropriate method of UA determination in plant raw material consists in simultaneous analysis of samples by means of HPLC and GC (or GC/MS) assays an overall peak of TTA and impurity compounds could be separated. If the separation of the peaks is impossible, we proposed to carry out methylation of phytogenous extracts containing UA and OA as an additional treatment with further fitting appropriate chromatographic conditions in order to separate the overall peak of UA and OA methyl esters.

When the mass extinction coefficient value (total light absorption of a substance solution with 1 g/L concentration along the optical path of 1 cm at the defined wavelength) for UA its methyl ester is known, the weights of these acids in a precisely weighed sample of the extract could be calculated from HPLC data basing on the assumption that the value for OA is the same as for UA The separation of the peaks corresponding to UA and OA in the gas chromatography analysis should allow to determine the mass ratio value for these acids according to the ratio of the areas of corresponding peaks. Thus, the simultaneous use of HPLC and GC methods could result in the obtaining of comprehensive and reliable information about qualitative and quantitative content of TTA in plant raw material under investigation.

Within the framework of the technique suggested we have determined the mass extinction coefficients at the wavelength of 210 nm for the reference samples of UA and its methyl ester of at least 98% purity; those values amounted to 9.3 and 5.6 arb. units/mg, respectively. In addition, chromatographic conditions were matched for UA and OA total peak separation from other components of plant raw material extracts. In order to simplify the composition of the extracts obtained from *Rh. adamsii*, the plant raw material was subjected to hydrodistillations before the extraction. Validity and reliability of the method suggested was demonstrated by the example of *Vaccinium praestans* Lamb. extract through the addition of precisely weighed UA sample. The data concerning raw material weight, the extracts obtained as well as UA and OA content are presented in Table 1.

From the Table 1 we notice that UA and OA content in *Rh. adamsii* is less than 0.1 %, and UA/OA ratio values for leaves and stems are close to each other. The total content of these acids in *Vaccinium praestans* (krasniqi) is higher, than in *Oxycoccus palustris* (cranberry); the contribution of UA to the total content of the acids in *Vaccinium praestans* is higher too. Thus, the press cake of *Vaccinium praestans* could be to recommended as a promising plant raw material for UA producing.

The results obtained demonstrate the possibility of use the suggested technique both for the extracts with low UA and OA content

TABLE 1

Quantitative content of ursolic and oleanolic acids in the plant raw material under investigation

Parameter	*Rhododendron adamsii*		*Vaccinium praestans*	*Oxycoccus palustris*
	Leaves	Stems		
Raw material mass, g	3.55	4.07	3.72	2.04
Mass of benzene extract, g	1.06	0.30	0.47	0.20
Sample of extract for HPLC analysis, mg	29.0	26.2	6.9	4.6
UA and OA mass in the sample*, mg	0.085	0.129	0.237	0.134
UA and OA mass scaled to raw material, mg	3.1	1.5	16.2	6.0
UA/OA ratio value**	2.2	2.5	4.7	4.1
UA and OA content in raw material, mass %	0.09	0.04	0.4	0.3

* According to HPLC data.

** According to GC data.

(*Rhododendron adamsii*), and for the extracts with high content of the acids (press cakes of berries). By means of the technique developed, UA and OA quantitative total content and mass ratio values in *Rhododendron adamsii* Rehd. leaves and stems, as well as *Oxycoccus palustris* and *Vaccinium praestans* Lamb. berries press cake have been determined.

CONCLUSION

In the present work a novel approach based on simultaneous use of HPLC and GC techniques has been suggested for the determination of the quantitative content of ursolic and oleanolic acids in plant raw material. Using the approach developed the content of these acids in *Rhododendron adamsii* leaves and stems and other two representatives of Ericaceae family such as *Oxycoccus palustris (*cranberry) and *Vaccinium praestans* (krasniqi) was determined.

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