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LIPIDS, LIPOPHILIC COMPONENTS, AND BIOLOGICALLY ACTIVE FRACTIONS OF *Viburnum opulus* L. SEEDS*

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Methyl and triterpenyl fatty-acid esters and triterpenic acids are isolated and identified from seeds of Viburnum opulus *(Caprifoliaceae). The biological activity of pigments and proteins is determined.*

Key words: *Viburnum opulus*, lipids, esters, sterols, triterpenes, fatty acids, triterpenic acids, protein, pigments.

We have previously studied the lipid composition of common viburnum (*Viburnum opulus*, Caprifoliaceae) seeds, a waste product of the food industry [1]. In continuation of this research, methyl and triterpenyl fatty-acid esters and certain lipophilic components were isolated and identified. The protein content and biological value in addition to the antioxidant activity of the pigments, the amount of which in viburnum seeds is comparable with that of lipids, were determined in order to evaluate the possibilities for multiple use of the seeds.

Esters were isolated from the neutral-lipid (NL) fraction [1] using repeated column chromatography (CC) and preparative TLC on silica gel. The content of methyl esters of fatty acids (MEFA) and triterpenyl esters (TTE) was 0.17 and 0.5%, respectively, of the air-dried (a/d) mass of starting material.

The IR spectra of MEFA and TTE contain absorption bands at 1740 cm^{-1}, which are characteristic of esters. The ^1H NMR spectrum of MEFA also exhibits a singlet at 3.6 ppm, which arises from a carbomethoxy group. Alkaline hydrolysis of MEFA produces 10 free fatty acids (FFA) (Table 1).

Total alkaline hydrolysis of TTE gave fatty acids (Table 1) and triterpenes (TTP). Preparative TLC in system 2 separated the TTP into TTP I and II. These gave a positive qualitative reaction for TTP [2]. The composition and identity of I and II were determined using chromatography—mass spectrometry. The mass spectra of decomposition products from I and II coincided with authentic samples.

According to the results, I is a mixture of sterols consisting of three components, the main one of which is β-sitosterol. Stigmasterol (or isofucosterol) and cholesterol were present in smaller quantities (Table 1). The distribution of bound and free [1] sterols in viburnum seeds differs in that the latter contains campesterol instead of cholesterol.

Mass spectrometric decomposition and literature data [3] identified in II triterpenes: three alcohols and possibly two ketones and three nonoxygenated triterpenes (Table 2). The two last groups of triterpenes may not be of natural origin. They may be formed during total alkaline hydrolysis or in the chromatograph vaporizer at temperatures >200ºC [4].

The presence of triterpene alcohols was confirmed by the preparation of their acetyl derivatives with subsequent characterization by chromatography—mass spectrometry. The separate components suggest that this group of compounds can be represented as α-amyrin > β-amyrin > lupeol.

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TABLE 1. Fatty-Acid Composition of MEFA (1), Triterpenyl Esters (TTE) from NL and MEFA (2), and FFA from Pigments of *Viburnum opulus* Seeds

Acids	MEFA (1)	MEFA (2)	TTE	FFA
11:0	-	Tr	-	-
12:0	Tr	0.6	0.4	0.4
13:0	-	Tr	-	-
14:0	0.8	1.2	1.2	0.7
15:0	Tr	0.9	Tr	0.2
16:0	14.1	28.2	62.2	16.5
17:0	Tr	6.7	-	0.8
18:0	1.8	4.0	3.4	2.4
18:1	55.3	40.6	27.1	26.2
18:2	23.6	16.1	2.0	49.5
18:3	3.3	1.2	-	3.3
20:0	-	0.5	3.7	-
21:0	1.1	-	-	-
Σ_{sat}	17.8	42.1	70.9	21.0
Σ_{unsat}	82.2	57.9	29.1	79.0

TABLE 2. Triterpenes Obtained After Hydrolysis of Esters of Triterpenes from *Viburnum opulus* Seeds

Terpenes	Content, %
Sterols - I	14.4[a]
β-sitosterol	93.6[b]
stigmasterol	5.3[b]
cholesterol	1.1[b]
Triterpenyl alcohols and their derivatives - II	29.3[a]
α-amyrin-urs-12-en-3β-ol	35.6[c]
β-amyrin-olean-12-en-3β-ol	12.3[c]
lupeol	Tr[c]
3-keto-urs-12-ene	20.0[c]
3-keto-olean-12-ene	6.9[c]
A:D-neoolean-12,14-diene	11.2[c]
A-neoolean-5,12-diene	4.8[c]

[a]Of the mass of triterpenyl esters; [b]of the mass of sterols; [c]of the mass of triterpenyl alcohols and their derivatives.

The ratio of separate alcohol components did not change after acetylation.

The pigment fractions were isolated in the next phase of the research. Lipid residues were determined to be present in them. The biological activity (antioxidant) of the pigments was verified. Thus, the pulp remaining after exhaustive removal of lipids was extracted with ethylalcohol. The yield of total pigments was 11.2% of the a/d mass of seeds. The concentrated extract was diluted with hot water and dissolved with gentle heating in order to separate them partially into the constituents. The resulting mixture was re-extracted successively with petroleum ether (1), diethylether (2), ethylacetate (3), and butanol (4). The yields of the corresponding fractions were: 0.1% (1), 1.9% (2), 2.9% (3), and 4.1% (4). The aqueous residue contained 2.2% of the seed mass. The petroleum-ether extract was oily and contained residual lipids according to TLC. The remaining fractions were powders. Nevertheless, fraction 2 contained (TLC) compounds that can be considered lipids or lipophilic components. These were isolated by separating fraction 2 into acidic and neutral products by treatment with 1% NaOH (the ratio of acidic and neutral products was about unity). The acidic products were separated by CC on silica gel. Three classes

of lipids were isolated: MEFA, FFA (control in system 3), and triterpenic acids (0.2, 0.35, and 0.4%, respectively, of the a/d seed mass) (Table 2). The remaining part contained unidentified components. The neutral fraction contained FFA and MEFA only in trace quantities. The triterpenic acids in the seed pigments were mainly ursolic acid (95.5% of the acid mixture by GLC) and a small quantity (4.5%) of oleanolic acid. Triterpenic acids were also identified in the glycolipid fraction, which has previously been hypothetically assigned to sterylglycosides. They were present in practically equal amounts: oleanolic (50.5%) and ursolic (49.5%) (GLC).

Only ursolic acid has previously been isolated from fruits of common viburnum [5].

Table 1 presents an analysis of the fatty-acid (FA) composition of all acyl-containing fractions of the lipids listed above. Table 1 shows that the FA composition of MEFA from the pigments is different from the NL composition in variety and total saturation (contents of 16:0 and 18:0 are doubled). The same trend is observed in the FFA isolated from pigments compared with FFA from NL [1]. However, the quantity of 16:0 is greater by four times.

A characteristic of the fatty-acid composition of MEFA **1** and **2** and TTE compared with the acyl-containing classes of NL and polarlipids from viburnum seeds is the fact that the principal unsaturated acid of these lipid classes is oleic and not linoleic.

The presence of MEFA and FFA in the diethylether extract of the pigments was unexpected. This may possibly be due to their release during separation from some complexes.

The fractions isolated by re-extraction from pigments, except the petroleum-ether extract, were checked for antioxidant activity by chemiluminescence, which is commonly used as a rapid analysis for testing synthetic and natural antioxidants [6, 7]. Only the ethylacetate fraction of the studied samples exhibited a noticeable inhibition. This may be due to the presence in it of hydroxycinnamic acid and its derivatives [5, 8], which might be potential oxidation inhibitors.

Table 3 presents the results of studying the antioxidant activity of the ethylacetate fraction by chemiluminescence.

The applicability of this method to a determination of the effectiveness of the natural antioxidants was checked using α-tocopherol and sunflower oil, which is known [9] to contain a mixture of tocopherols. The rate constant [$K_7 = (2.8 \pm 0.2)\cdot 10^6$ L/mol·sec] for reaction of α-tocopherol with peroxide radicals of ethylbenzene was determined from experiments with α-tocopherol; with sunflower oil, $K_7 = 2.5\cdot 10^6$ L/mol·sec ($K_7 = 2.6\cdot 10^6$ L/mol·sec, [10]). The content of α-tocopherol in sunflower oil, which was established by the method presented above, is $1.9\cdot 10^{-3}$ mol/L. This agrees well with the literature data [9], $1.4\cdot 10^{-3}$ mol/L (70 mg%).

An investigation of the kinetics indicates (Table 3) that the ethylacetate extract possesses antioxidant activity. The extract contains 4% antioxidant. The inhibition of the extract is rather high ($K_7 = 10^5$ L/mol·sec, compared with $K_7 = 2.4\cdot 10^4$ and $2.6\cdot 10^6$ L/mol·sec for ionol and α-tocopherol, respectively). The kinetics for antioxidant consumption are typical of the presence of at least two inhibitors with different effectiveness K_7^1 and K_7^2 (K_7^1 and K_7^2 are the rate constants for the reaction of peroxide radicals of ethylbenzene with these inhibitors). The constants K_7^1 and K_7^2 vary depending on the extract concentration.

Therefore, the mechanism for the reaction of ethylbenzene peroxide radicals with antioxidants in the studied extracts is more complicated than that for α-tocopherol.

Protein was isolated from seed powder by alkaline extraction. The yield of lipid—protein was 16-18% of the initial mass. The extract contained 28-29% protein.

The amino-acid composition suggested that the protein from common viburnum seeds contained 15 amino acids. Of these, half are essential: threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, and lysine (Table 4).

The amount of amino acids such as lysine that is deficient in natural sources is at least twice greater then in the amount recommended by FAO/WHO (World Health Organization).

The biological value of the extract was verified in infusions [11] and compared with fresh lyophilized milk. The value was 64%.

Thus, the results and previous investigations [1] of the chemical composition of common viburnum seeds show that the seeds are a valuable source of such biologically active components as lipids, pigments, and proteins.

TABLE 3. Antioxidant Activity of the Ethylacetate Fraction of Pigments from *Viburnum opulus* Seeds

No.	$Wi \cdot 10^8$***, mol/L·sec	Concentration of extract in reactor, $mg/mL \cdot 10^2$	$K_7^1 \cdot 10^{-6}$	$K_7^1 \cdot 10^{-5}$	$K_7^2 \cdot 10^{-5}$
			L/mol·sec		
1*	4.4	3.7	2.8	-	-
2**	3.8	17.7	2.5	-	-
3	3.8	1.43	-	5.6	2.6
4	3.4	1.77	-	4.3	2.3
5	3.7	2.76	-	2.5	2.1
6	3.7	4.88	-	1.5	1.5

*α–Tocopherol; **sunflower oil; ***initiation rate. The uncertainty in K_7 is less than ±10%.

TABLE 4. Amino-Acid Composition of Lipid—Protein Isolate from *Viburnum opulus* Seeds

No.	Amino acid	%, mol	%, wt
1	Aspartic acid	10.43	6.0
2	Threonine	4.52	2.3 (2.8)
3	Serine	7.3	3.3
4	Glutamic acid	22.60	14.3
5	Proline	6.84	3.4
6	Glycine	10.20	3.3
7	Alanine	7.65	2.9
8	Valine	4.87	2.5(4.2)
9	Methionine	-	- (2.2)
10	Isoleucine	4.87	2.8(4.2)
11	Leucine	10.55	6.0(4.8)
12	Tyrosine	10.43	8.2(2.8)
13	Phenylalanine	9.97	7.1(2.8)
14	Histidine	5.45	3.6
15	Lysine	14.72	9.3(4.2)
16	Arginine	3.94	3.0

The table does not include tryptophane and cysteine. Recommendations of FAO/WHO are given in parentheses.

EXPERIMENTAL

^{1}H NMR spectra were recorded on a Bruker AM-300 instrument in $CDCl_3$ with TMS internal standard. IR spectra were obtained on UR-20 and Specord M-80 spectrometers; mass spectra, on a computerized HP-5890 chromatograph—mass spectrometer with an HP 5972 A mass-selective detector. The analytical conditions were: capillary quartz column Ultra-250 m × 0.2 mm, bound phase 5% $PhMeSiO_2$, initial temperature 30°C, temperature gradient 10°/min, final temperature 300°C, carrier gas flow rate 1 mL/min, vaporizer temperature 305°C. Spectra were scanned at 1 spectrum/sec. The scanning range was 39-500 amu. Data were processed using ChemStation HPMS. Mass spectra were integrated using a database and spectrum—structure correlations.

GLC analysis of MEFA was performed as reported earlier [1]. Methyl esters of triterpenic acids were analyzed on a Chrom-5 chromatograph (1.2×3 mm column, 1% OV-17 on Chromosorb W, 230°C, He carrier gas 80 mL/min).

Analytical TLC was performed on Silufol plates using solvent systems petroleum ether—diethylether (9.5:0.5) (the

system was used for preparative TLC of MEFA and TTE) (1), 5:5 (2), 8:2 (3). Preparative TLC was carried out on silica gel with 10% gypsum on 15×15 cm glass plates in the above solvent systems. Free triterpenic acids were isolated by CC on silica gel (100/160 mesh) from the polar-lipid fraction using $CHCl_3$—CH_3OH solvents from 0 to 50%; NL, from the pigment fraction using C_6H_6—$CHCl_3$ from 0 to 50%.

Alkaline Hydrolysis of Triterpenic Esters. TTE (100 mg) was treated with KOH (5 mL, 20%) in CH_3OH and refluxed for 20 h. The course of the reaction was monitored by TLC using solvent system 1. The reaction mixture was diluted three times with water and extracted three times with diethylether. The ether extracts were evaporated to give solid triterpene compounds (50 mg). The mother liquor was acidified with H_2SO_4 (10%). The acidic solution was extracted three times with diethylether. The ether extracts from the acidic solution contained fatty acids (20 mg) with a slight impurity of triterpenes. The triterpenes were separated using preparative TLC in solvent system 3 and combined with the previous TTP fraction. The combined TTP fraction was separated using preparative TLC in solvent system 2 into TTP I (14.4 mg) and TTP II (29.3 mg), which were recrystallized from CH_3OH and analyzed by chromatography—mass spectrometry.

The triterpene alcohols were acetylated by acetic anhydride in pyridine at room temperature.

The antioxidant activity of the extracts was determined using the model reaction of liquid-phase initiated oxidation of ethylbenzene. The initiator was azoisobutyronitrile. The luminsecence activator was dibromoanthrene. A solution of pigment extract in ethylbenzene was placed in a thermostatted (60°C) oxidizing solution of ethylbenzene (initial volume V_0 = 5 mL) so that its concentration in the reactor was $(1.4\text{-}5)\cdot 10^{-2}$ mg/mL.

The experimental data were processed according to the literature method [12, 13] in order to determine the rate constant K_7 and the antioxidant concentration in the studied extract.

The lipid—protein fraction was obtained by extracting seed powder with NaOH (0.2 N) for 30 min at room temperature (powder—extractant ratio, wt/vol, 1:10). The insoluble solid was separated by centrifugation. The supernatant was acidified to pH 5.3. The pale red precipitate that formed was separated by centrifugation at 8000 rpm for 20 min. The precipitate was washed with distilled water, centrifuged under the same conditions, and lyophilized.

The content of dry protein was determined by the Kjeldahl method [14]; of proteinaceous nitrogen, by the Lowry method [15]. The amino-acid composition was determined after hydrolysis by HCl (5.7 N) for 24 h on an AAA 331 (Czech Republic) amino-acid analyzer.

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