Research Article

Potential Effective Lipid-Lowering Medicine - Betulonic Acid. Mild Selective Synthesis and Pharmacological Activity

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ABSTRACT

The practical synthesis of betulonic acid, suitable for pharmaceutical industry, from betulin in 93-97 % yield by $K_2Cr_2O_7 – H_2SO_4$ as an oxidant using $H_3[PW_{12}O_{40}]$, $H_7[P(Mo_2O_7)_6]$ and $H_4[SiMo_{12}O_{40}]$ in aqueous acetone at 15-25°C has been developed. High lipid- and cholesterol-lowering activity of betulonic acid in the medium of pumpkin seed oil has been estimated.

Keywords: betulin, oxidation, betulonic acid, heteropoly acids, lipid- and cholesterol-lowering.

INTRODUCTION

Betulonic acid, a pentacyclic triterpenoid lupanes series, possesses several biological properties such as antiviral, antitumor, anti-inflammatory, hepatoprotective as well as immunostimulant activities.[1-3] In addition, betulonic acid is an important starting material for the synthesis of other betulin derivatives (oximes, amides, esters etc.). Biotransformation of betulinic acid – well-known antitumor substance, resulted in formation of betulonic acid and both acids give the same metabolites.[4]

Some studies (Sorokina I.V. 2004-2010) proved hepatoprotective and regenerative effects of betulonic acid and its derivatives in the liver cell (hepatocytes, sinusoids endothelial cells, Kupffer cells) in different tumor diseases. Betulonic acid and its β-alanilamide exhibited polytarget effect on liver cell populations, potentiating cytotoxic effect in some cells and stimulating regenerative reactions in other cells, when the treatment was carried out in combination with cytostatic. Recovery of hepatocyte ultrastructure in combined use of cytostatics and betulonic acid and its derivatives was faster.[5-7]

Well-known syntheses of betulonic acid are based on the oxidation of betulin **1** using reagents containing Cr(VI) at low temperatures, for an example Jone's reagent ($CrO_3 – H_2SO_4$ - acetone). A 75% yield was achieved after difficult purification by multiple recrystallization, fractional extraction by using very large volumes of solvents.[8-12]

The main problems in the oxidation of 1 are due firstly to non-selectivity of the process, because this molecule contains three active centers: the primary (at C-28) and the secondary (at C-3) alcohol groups and isopropylidene moiety. Secondly, it is difficult to regulate the range of oxidation, because the products may be aldehydes or ketones as well as acids. Thirdly, the labile state of **1** structure makes conditions for different rearrangements as follows on the basis NOESY-spectra calculation taking into account Overhauzer's effect.[13,14]

Earlier we showed the possibility of practical synthesis of betulonic acid **2** using selective oxidation of **1** on aluminium solid support.[15] Synthesis of **2** with a minimum amount of impurities is particularly important for medicinal substances. It was proposed the selective oxidation up to **2** was due to the influence of Al^{3+}-ions as Lewis acid.[15]

In this paper, firstly, we continued to study the effective methods of synthesis **2** as a potential drug. Secondly, we have learned lipid- and cholesterol-lowering activity of **2** in the experiment on rats.

We wish to describe a simple procedure that can be used for practical synthesis of **2** from **1** in quantitative yield using $K_2Cr_2O_7 – H_2SO_4$ as an oxidant in the presence of Lewis acids - heteropoly acids of Keggin's structures derivatives of type 1:12 such as $H_3[PW_{12}O_{40}]$, $H_7[P(Mo_2O_7)_6]$ and $H_4[SiMo_{12}O_{40}]$ at room temperature**.**

It has been shown, that catalytic activity of heteropoly acids (heteropolymetallates) in oxidation and hydration processes is due to including transition metal ions with d^0-configuration (e.g. Mo(VI) and W(VI)) likewise Brensted's and Lewis's acidities.[16,17] In particular, the efficient method for the oxidation of some alcohols with 34% hydrogen peroxide catalyzed by $H_3[PW_{12}O_{40}]$[18] was described**.** This fact allows considering the simple polyoxometallates as potential redox catalysts in the oxidative transformation of **1.**

MATERIALS AND METHODS

Betulin 1 ($C_{30}H_{50}O_2$) was prepared according to the authors[19] and recrystallized from 2-pronanol; **M.p.** 260° (lit. 254-256°C)[16]; purity 99.5 % by **HPLC**. **IR spectrum** : (KBr) ν, cm^{-1}: 3470 st (OH), 1640 st (C=C). **^{1}H NMR** (DMSO-d_6) δ, ppm: 4.67 m (1H, $=CH_2$), 4.57 m (1H, $=CH_2$), 3.78 br. s (1H, 28-CH_2OH), 3.31 m (1H, 28-CH_2OH), 3.17 m (1H, 3-

CHOH), 2.36 m (1H, 19-CH), 1.66 s (3H, CH_3), 1.23 s (3H, CH_3), 0.96 s (3H, CH_3), 0.94 s (3H, CH_3), 0.80 s (3H, CH_3), 0.74 s (3H, CH_3). **^{13}C NMR** (DMSO-d_6) δ, ppm: 76,71 (C-3), 109,46 (C-29), 150,24 (C-20), 57,87 (C-28). **EI-MS** (*m/z*, %): 442 (M+, 40), 411 (60), 203 (95), 189 (100), 95 (85).

Preparation of heteropoly acids solution. Heteropoly acids (Aldridge, Germany) were used.

Synthesis of betulonic acid 2. To the white suspension of betulin **1** (ultra-sound dispersed, 0.5 g, 1.1 mmol in 46 mL acetone) in sequence solution of heteropoly acid (Table 1), oxidizing reagent ($K_2Cr_2O_7$, 1g, 3.3 mmol), water (6.6 mL) and conc. H_2SO_4 (1.6 mL) at 15-25°C were added. The reaction mixture was stirred during 1.5-3.0 hours at these temperatures (HPLC-control). After completion of the reaction (as monitored by HPLC) water (250 mL) was added. Crude betulonic acid **2** was floated (in white flakes) and after filtration **2** was multiple washed by hot water. The solid (0.18g) was recrystallized from methanol. The crystalls were isolated and dried in a vacuum oven to afford pure **2** (> 95 % by **HPLC**), **M.p.** 250 – 252ºC(methanol) (lit. 245-248 ºC).[20] **IR spectrum** : (KBr) *ν*, cm^{-1}: 1705 st (C=O), 1641 st (C=C), 883 st (=CH_2). **^{1}H NMR** (DMSO-d_6) δ, ppm: 4.68 s (1H, 29-H), 4.55 s (1H, 29-H), 2.23 m (1H, 19-H), 1.65 s (3H, 30-CH_3), 1.02–1.95 (3H, complex, CH_2, CH), 1.02 s (3H, 26-CH_3), 1.00 s (3H, 25-CH_3), 0.98 s (3H, 27-CH_3), 0.86 s (3H, 23-CH_3), 0.85 s (3H, 24-CH_3).**^{13}C NMR** (DMSO-d_6) δ, ppm: 216.52 (C-3), 109.67 (C-29), 150.33 (C-20), 177.26 (C-28). **EI-MS** *m/z* (%): 454 (M+, 58), 248 (64), 219 (42), 205 (76), 189 (88), 136 (100), 121 (90).

Betulonic aldehyde as a standard was prepared according to the authors [15], **M.p.** 163–165 °C (lit. 165–166 °C).[17] **IR spectrum** : (KBr) *ν*, cm^{-1}: 1730–1728 st (C=O), 1641 st (C=C); 883 st (=CH_2). **^{1}H NMR** (DMSO-d_6) δ, ppm: 9.67 s (1H, 28-CHO), 4.68 s (1H, 29-H), 4.55 s (1H, 29-H), 2.99 dd (1H, 3α-H), 2.23 m (1H, 19-H), 1.65 s (3H, 30-CH_3), 1.02–1.95 (3H, complex, CH_2, CH), 1.02 s (3H, 26-CH_3), 1.00 s (3H, 25-CH_3), 0.98 s (3H, 27-CH_3), 0.86 s (3H, c, 23-CH_3), 0.85 s (3H, c, 24-CH_3). **^{13}C NMR** (DMSO-d_6) δ, ppm: 216.52 (C-3), 109.67 (C-29), 150.33 (C-20), 206.55 (C-28). **EI-MS** *m/z* (%): 438 (11,5), $[M\text{-}CHO]^+$ 409 (20.0), 273 (3.2), 219 (20.2), 205 (38.4), 189 (39.6), 133 (35.6), 105 (55.8), 81 (65), 55 (100).

Methods of Carbonyl Value (CV) Determination: hydroxylamine hydrochloride (4 g) was dissolved in 8 mL of water and diluted with 80 mL of ethanol. 60 mL of 0.5 M ethanol solution of KOH and 10 mL of bromophenol blue solution were added under stirring and then the mixture was quickly filtered.

2 g of the keto-group containing compound and 75 mL of hydroxylamine solution were added to the flask, then the mixture was refluxed for 1 hour in a water bath. After cooling the base excess was titrated by 0.5 M HCl until the purple colour became yellow. At the same time the control experiment was performed. The difference between the two definitions refers to the number of hydroxylamine that were spent on oxime-formation of C=O group. 1 mol of KOH is equivalent to 1 mol of hydroxylamine, so the quantity of KOH is a measure of content of keto-group in the compound.[21]

$$CV = \frac{(V_x - V_n) \times 28{,}05}{a},$$

Where V_x - the volume of HCl used on titration of the control solution, mL; V_n - the volume of HCl used on titration of the test solution, mL; *a* – mass of sample, g.

Pumpkin seed oil (state standard 42-8110-06), thymol (specifications 6-09-37-36-79).

Biomedical Research

Animals

Male white nonlinear 1-2 months old rats weighing 220-270 g were used for this study. The rats were housed in standard polycarbonate cages on substyles area 2150 cm^2, 5- animals per cage, under controlled conditions of temperature (18-24°C), relative humidity (30-70%) and 12 h light/dark cycle (06.00-18.00 - day 18.00-06.00 - night), with free access to commercial pellet diet and water. Acclimation period - 14 days.

Content of natural antioxidants in Pumpkin (*Cucurbita Pepo*) Seed Oil: α-tocopherol (6 mg%), mixture γ-tocopherol and γ-tocotrienol (near 50 mg%), carotinoids (0,6 – 1 mg%) and phytosterols, among them β-sitosterol (100-150 mg%) was estimated by UV-Vis – spectroscopy and by RP- HPLChromatography.[22]

The formulation: *the pharmaceutical composition (wt%):* betulonic acid – 0.5; thymol – 0.1, pumpkin seed oil (PSO) at 100.0.

The composition was prepared by dissolving 0.5 g of betulonic acid **2** (85-90% purity) and 0.1 g of thymol in PSO. After stirring the resulting mixture was dispersed by ultra-sound to dissolve the solid components of the pharmaceutical composition.

White rats had been induced experimental hypercholesterolemia by intraperitoneal administration of Tween-80.

The oil solution of the investigated pharmaceutical composition was orally administered to experimental groups of animals simultaneously with Tween-80.

The control group of animals received 0.5 mL of water instead of the test substances. 12 hours after intraperitoneal injection of Tween-80 the rats were sacrificed by decapitation.

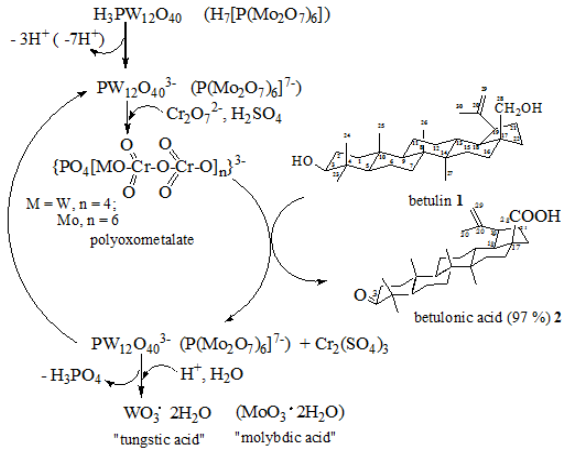
The content of cholesterol and triglycerides was estimated in blood serum.

The concentrate of triterpene acids was used as a comparative pharmaceutical composition. It had been derived from sea buckthorn berries meal and mainly presented by triterpene acids, small amount of organic acids and other impurities.

RESULTS AND DISCUSSION

Synthesis of Betulonic Acid **2**

It was shown that the addition of oxidant system $K_2Cr_2O_7$–H_2SO_4 in the presence of heteropoly acids to the suspension of betulin **1** in water-acetone medium initially led to the appearance of flakes of inorganic nature soluble in water, which composition corresponded to general formula $Cr_2(SO_4)_3$ x H_2SO_4 yH_2O. The impurity of elements P, Mo, W or Si in the flocculated sediment was detected by atomic absorption spectrometry. Colourless flakes obtained green in 15 - 25 minutes, but supernatant liquid became clear and homogeneous. Isolation of the product - betulonic acid **2** in quantitative yield performed in 1.5 - 3 hours by adding water to the separated supernatant (Scheme 1). It was noted that the crude does not require complicated purification.[23]



Scheme 1. Oxidation of **1** by $K_2Cr_2O_7$ in water-acetone medium in the presence of heteropoly acids

It is necessary to comply with conditions: *i* – the oxidants threefold molar excess to substrate **1**; *ii* – weight ratio acetone: water in reaction mixture must be no less 6:1; *iii* – the temperature range is 15-25°C. Concentration of heteropoly acid may be decreased up to $5 \cdot 10^{-9}$ - $5 \cdot 10^{-8}$ mol·L^{-1}, if betulin concentration is about 0.2 mol·L^{-1}.

Chromium-containing complexes may be converted into chromium (III) sulphate and may be regenerated into heteropoly acids after oxidation of **1**. The end of the reaction by adding a large amount of water to the reaction mixture led to difficulty in getting soluble "tungstic" or "molybdic" acids that are capable to the regenerated by the action of H_3PO_4 (UV-vis monitoring) according to Scheme 1.

Consequently, heteropoly acids also promote the protection of isopropenyl group.

The data in Table 2 indicate the possibility of controlling the depth of oxidation in time.

Thus the oxidation of **1** in the presence of heteropoly acids for 10 - 60 minutes resulted in **2** with a low yield of 48 - 75% (CV = 110, slight decrease of the melting temperature) and the presence of betulonic aldehyde in the precipitate (Table 1, IR - and NMR - data). The increasing of time of **1** oxidation to 3 hours increases the selectivity and the yield of product **2** to 92 - 98% (CV = 70, IR - and NMR - data).

The increase in selectivity of oxidation in the presence of Lewis acid - $Al_2(SO_4)_3$ and $ZnSO_4$, as well as heteropoly acids, is probably caused by increasing the homogeneity of the reaction.

Venturello[24] using crystallography techniques characterized polyperoxometallate $\{PO_4[W(O_2)_2]_4^{3-}\}$ as an active oxygen transfer agent in the oxygenation of organic compounds by hydrogen peroxide catalyzed in the presence of $H_3[PW_{12}O_{40}]$. Similarly as reported by R. Tayebee and M.H. Alizadeh,[15] we propose that oxidation of **1** by $K_2Cr_2O_7$ – H_2SO_4 in aqueous acetone in the presence of heteropoly acids proceed with participation of polyoxometallates containing Cr^{+6}. Heteropoly acids of Keggin's structure type 1:12 are able to be as ligands for transition metal ions[12,13] and to give more soluble polyoxometallates (Scheme 1).

On the other hand the increasing of homogeneity in the reaction zone may be due to the formation of complexes of **1** with Lewis acid.

A new intensive band in the region of 300 - 330 nm appeared in UV-spectra of betulin acetone solution in the presence of Lewis acids - heteropoly acids or $Al_2(SO_4)_3$ and $ZnSO_4$, while **1** and its oxo-derivatives ethanol solutions without Lewis acids absorbed in the region of 190 - 220 nm (Figure 1 a). Absorbance of **1** solution with H_2SO_4 and $Al_2(SO_4)_3$ is noticeably lower (curves 1 and 2, Figure 1 a) and it is significantly increasing during the time (Figure 1 b). The shape and the position of the absorption band of **1** solution in the presence of heteropoly acids in the UV - visible spectrum is practically independent of the nature of heteropoly acids and of dissolving by adding small amounts of H_2SO_4. Besides the absorbance of **1** due to $H_7[P(Mo_2O_7)_6]$ was 3-fold more intensive than the ones in the presence of $Al_2(SO_4)_3$.

The appearance of a new band at 300 - 330 nm in the **1** spectra in the presence of Lewis acids probably indicate the formation of complexes of 1 with acids in solution.

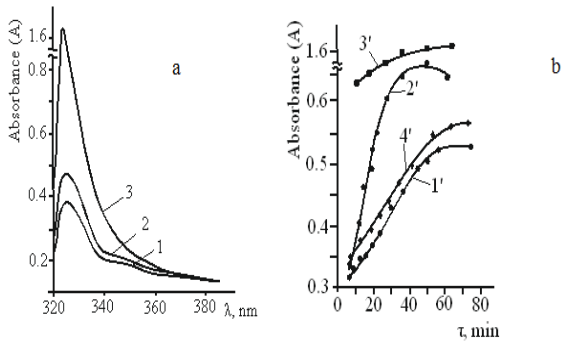


Figure 1: UV - visible spectra of 4,4 · 10^{-5} M betulin acetone solutions **a)** in the presence of acids, τ=30 min; **b)** the change of A_{330} absorbance of 4,4 · 10^{-5} M betulin acetone solutions in time A = f (τ)

№	H_2SO_4	$Al_2(SO_4)_3$	$H_7[P(Mo_2O_7)_6]$
1, 1'	$1,0\cdot10^{-3}$ M	–	–
2, 2'	–	$7,0\cdot10^{-6}$ M	–
3, 3'	–	–	$3,3\cdot10^{-6}$ M
4'	$1,0\cdot10^{-3}$ M	$7,0\cdot10^{-6}$ M	–

The influence of heteropoly acids on UV-spectrum of **1** reaction mixture containing oxidant system ($K_2Cr_2O_7$–H_2SO_4) is difficult to estimate because oxidant absorbs in the same region of 298 nm. The feature of the oxidant spectra is multiple band in the region of 350-360 nm. Comparison of UV - spectra of the reaction mixture "betulin - oxidant ($K_2Cr_2O_7$–H_2SO_4 – $H_3PW_{12}O_{40}$)" and oxidant solution ($K_2Cr_2O_7$–H_2SO_4 – $H_3PW_{12}O_{40}$) at the same oxidant concentration shows the significant absorbance increase in the spectrum of reaction mixture (Figure 2).

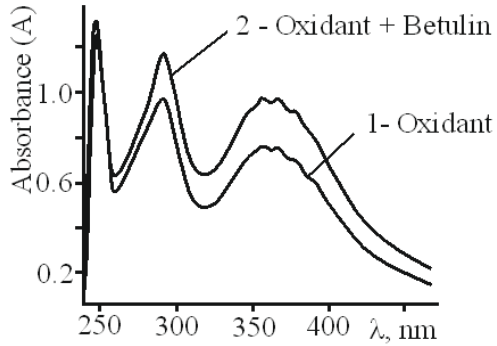


Figure 2: UV - spectra of the mixtures, τ = 5 min: curve 1 – "oxidant ($K_2Cr_2O_7$–H_2SO_4) – $H_3PW_{12}O_{40}$ – acetone"; curve 2 – "betulin - oxidant ($K_2Cr_2O_7$–H_2SO_4) – $H_3PW_{12}O_{40}$ – acetone".

These data may characterize the ability of the metal and oxygen orbitals to overlap and form π – bond (M = O, M= Mo, W, Cr, Al etc.) in the reaction of **1** with heteropoly acids, $Al_2(SO_4)_3$ and Cr^{6+} and the formation of polyoxometallates as intermediate structures in the reactions with participation of heteropoly acids according to Scheme 1.

Heteropoly acids containing an element with d^0 - configuration may possibly act as ligands in dichromate oxidant structures. In contrast to dichromate-anion polyoxometalates are capable to react with isopropenyl group by tungstate - or by molybdate - ion moieties. It prevents the direct exposure of a strong oxidant Cr^{6+}.

Thus, the oxidation of **1** to **2** by $K_2Cr_2O_7$ – H_2SO_4 reagent in aqueous acetone in the presence of heteropoly acids such as $H_3[PW_{12}O_{40}]$, $H_7[P(Mo_2O_7)_6]$ and $H_4[SiMo_{12}O_{40}]$ offers several advantages, including simplicity of operation**,** easy work-up, high yield and high selectivity. The main advantage of this procedure is possibility to obtain larger amounts of **2** at 15-25°C.

Lipid- and Cholesterol-Lowering Activity of Betulonic Acid

Estimation of betulonic acid lipid- and cholesterol-lowering activity has been studied both in pure substance **2** and as an active component of the pharmaceutical composition in pumpkin seed oil (PSO). PSO was chosen as a medium because it is rich in natural antioxidants such as α-tocopherol, mixture γ-tocopherol and γ-tocotrienol, carotinoids, phytosterols, among them β-sitosterol and high content of unsaturated acids (up to 80%), that due to the use of PSO as a lipid metabolism regulator. PSO shows a good result in the treatment of lipid-associated diseases.[25] It was assumed, that the hypocholesterolinic effect of PSO is due to the structure similarity of β-sitosterol with cholesterol, because β-sitosterol may displace cholesterol from low density lipoproteins (LDL).[26,27]

The other mechanism of β-sitosterol action is the formation of sufficiently stable complexes with cholesterol that makes difficult removal of the essential lipids from corneous layer.[26] Common action by unsaturated acids and by phytosterols promotes disorder correction of lipid metabolism, too.[28]

The experiments showed (Table 3), that the level of cholesterol in the rats blood of the control group significantly increased (292.9%) in comparing with intact animals as a result of administration of Tween-80. The administration of pharmaceutical composition with **2** decreased cholesterol in animals' blood under the action of Tween-80.

Table 1: The ratio of components of heteropoly acids solution

	Heteropoly acids		
	$H_3[PW_{12}O_{40}]$	**$H_7[P(Mo_2O_7)_6]$**	**$H_4[SiMo_{12}O_{40}]$**
m, g (mmol)	0.15 (0.052)	0.15 (0.081)	0.06 (0.033)
acetone, mL	5	10	10
water, mL	0.1	-	1
conc. H_2SO_4, mL	0.1	1	1
Colour of transparent solution	colourless	green	orange-brown

Table 2: The dependence of yield **2** on oxidation time of **1** in aqueous acetone by system $K_2Cr_2O_7 – H_2SO_4$ in the presence of heteropoly acids

№	Heteropoly acid	τ, min	CV, carbonyl value *	m.p., ^{0}C	Yield **2**, %
1	$H_3PW_{12}O_{40}$	60	110	220 – 225	75
2		120	75	245 – 246	85
3		180	70	251 – 252	97
4	$H_7[P(Mo_2O_7)_6]$	90	73	249 – 250	92
5		180	70	250 – 252	98
6	$H_4[SiMo_{12}O_{40}]$	10	110	166 – 167	48
7		90	67	246 – 247	90

* $CV_{theor.}$ = 70 for compounds with one carbonyl group (betulonic acid); $CV_{theor.}$ = 140 for compounds with two carbonyl groups (betulonic aldehyde)

Table 3: The effect of the pharmaceutical composition and triterpene acids concentrate, derived from sea buckthorn berries meal, on the cholesterol content in the experimental animals' blood.

Group	Cholesterol content, mmol/L		Result, %
	Tween-80	Tween-80 + composition	
Intact animals, n=5	3,10±0,67*	-	-
Control, n=5	12,18±0,64	-	+292,9
Triterpene acids concentrate, n=5	-	9,11±0,71	-25,2
Betulonic acid, n=5	-	8,75±0,58	-28,2
Pharmaceutical composition, n=5	-	7,32±0,80	-39,9

*without administration of Tween-80

Table 4: The effect of the pharmaceutical composition and triterpene acids concentrate, derived from sea buckthorn berries meal, on the triglycerides content in the experimental animals' blood

Group	Triglycerides content, mmol/L		Result, %
	Tween-80	Tween-80 + composition	
Intact animals, n=5	0,64±0,15*	-	-
Control, n=5	0,88±0,15	-	+27,3
Triterpene acids concentrate, n=5	-	0,59±0,15	-32,9
Betulonic acid, n=5	-	0,52±0,17	-40,9
Pharmaceutical composition, n=5	-	0,44±0,14	-50,0

*without administration of Tween-80

The results in Table 3 show that the cholesterol-lowering effect of the inventive pharmaceutical composition exceeded the action of triterpene acids concentrate.

Lipid-lowering effect was determined by the content of triglycerides in plasma (Table 4).

As can be seen from the data presented in Table 4, the use of the pharmaceutical composition greatly reduces the level of triglycerides in the blood compared with triterpene acids concentrate.

Lipid-lowering activity of betulonic acid probably due to its interaction with cholesterol is similar to β- sitosterol. It is known that cholesterol in the presence of betulin increases antitumor effect.[29]

The pharmaceutical composition may be included in gelatin capsules and used for the treatment and prevention of a wide range of metabolic disorders, including lipid-related diseases (diabetes, arteriosclerosis, varicose veins, hepatosis).

From these results it can be concluded that the proposed pharmaceutical composition with **2** provides high efficiency, bioavailability, non-irritating and doesn't have other side effects.

CONCLUSION

A mild selective synthesis of betulonic acid by betulin oxidation in the presence of heteropoly acids in aqueous acetone at room temperature has been developed. Synthesis of betulonic acid by new methods allows getting a minimum amount of such impurities as betulonic and betulinic aldehydes, isoprenyl moiety oxidation products. It is very important for medicinal substances.

Lipid- and cholesterol-lowering effect of pharmaceutical composition with betulonic acid and thymol in the medium of pumpkin seed oil has been estimated.

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