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Antioxidant activity of silybin and 2,3-dehydrosilybin from *Silybum marianum* (L). Gaertn. Fruits

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Abstract
Silybin (1) and 2,3-dehydrosilybin (2) were isolated from fruits of milk thistle [*Silybum marianum* (L). Gaertn.] cultivated in the Samara region (Russian Federation). 2,3-dehydrosilybin for the first time is described for the given plant, cultivated in Russian Federation. Silybin and 2,3-dehydrosilybin and also the fluid extract of *Silybum marianum* fruits (the ratio of the fruits and 80% ethanol is 1:1) have expressed antioxidant activity, decreasing the level of the lipid peroxidation caused $FeSO_4$ in the homogenate of the liver rats. The antioxidant activity of 2,3-dehydrosilybin is reported for the first time and on 14,0% above those of silybin.

Keywords: milk thistle, *Silybum marianum* (L). Gaertn., fruits, flavolignans, silybin, 2,3-dehydrosilybin, antioxidant activity.

1. Introduction
The drugs from the fruits of the milk thistle [*Silybum marianum* (L). Gaertn. Family *Asteraceae*] (Legalon, Carsil, Silybinin, Silymar, fluid extract etc.) are widely used as the effective hepatoprotective pharmaceuticals [1–7]. The preparations of the *Silybum marianum* fruits have ability to inhibit a lipid peroxidation in a tissue of the liver and to increase the activity such antioxidant enzymes, as superoxiddismutase and catalase [8]. The unique hepatoprotective activity of preparations from *Silybum marianum* fruits is caused by flavanonollignans, among which most typical are silydianin, silychristin and silybin (1) [1-7, 9] and last compound is offered by us as a State standard sample for the purposes of standardization of drugs and remedies of the given plant [6, 10]. The mixture of silydianin, silychristin and silybin (1) is named by "silymarin" [2, 5], which used as the substance for development of the hole series of hepatoprotectors. There was shown later the activities of another flavanolignans and namely silandrin, silyhermin and silymonin, which are desoxyanalogues of silybin, silychristin and silydianin, respectively [1]. According to our results [11] in the *Silybum marianum* fruits in appreciable amounts contains 2,3-dehydrosilybin (2) (Fig. 1), which for the first time has been by us isolated from *Silybum marianum* cultivated in Russian Federation. Earlier 2,3-dehydrosilybin (2) has been isolated from *Silybum marianum* fruits only with Indian scientists [12] and also was synthesized by means of dehydrogenation of natural silybin (1) [13]. Taking into account this fact, 2,3-dehydrosilybin was of interest in the plan of the study of the biological activity. In the present time we report the comparative study of the antioxidative actions of the 2,3-dehydrosilybin, silybin and *Silybum marianum* fluid extracts prepared according to our method [14].

2. Materials and Methods
2.1. Extraction of Plant Materials
The isolation of silybin (1) 2,3-dehydrosilybin (2) (Fig. 1) was carried out from the fruits of *Silybum marianum* (L.) Gaertn. cultivated in the Samara region (Russian Federation). The date of collection of plant material is 20 August 2014. The plant identified by PhD. Vitaliy Ryzhov. The voucher number of investigated plant is 570. The voucher sample was kept in herbarium of the Department of Pharmacognosy of Samara State Medical University. The extraction of the flavolignans from the fruits of *Silybum marianum* (200,0 g) there were carried out with the using of 80% EtOH at the heating. The obtained extract was evaporated and the residues were dried on a polyamide "Woelm". This dried extraction there was used for the preparation of compounds (1) and (2) by means of the column chromatography on polyamide "Woelm" (8x20 cm) (solvent gradient systems: water/EtOH).

2.2. Isolation of Compounds from Plant Materials

Compounds (1) and (2) were eluated by the using of chromatographic systems water/EtOH 6:4 and 3:7 respectively. The subsequent rechromatography of these fractions on silica gel L 40/100 (4x20cm) (solvent gradient systems: chloroform/MeOH) resulted in two flavolignans: silybin (1) (300 mg) (by use of chloroform/MeOH 98:2) and 2,3-dehydrosilybin (2) (50 mg) (chloroform/MeOH 93:7). The fluid extract (1:1) from the fruits of *S. marianum* was prepared by our method [14] by means of extraction of thus drugs with 80% EtOH.

2.3. Methods of Structural Elucidation and Analysis of Plant Materials

^{1}H-NMR spectra were measured on spectrometer "Bruker AM 300" (300 MHz), mass spectra were determined on a mass spectrometer "Kratos MS-30", the recording of the UV spectra were performed using a spectrophotometer "Specord 40" (Analytik Jena). The optical rotation data of compounds were determined on a Polarimeter "Polamat A". Melting points were determined on a Boetius melting points apparatus. Chromatographic plates "Sorbfil PTSKH-AF-A-UV" were used for TLC, solvent systems: $CHCl_3$-MeOH-H_2O (26:14:3) and chloroform–acetone-formic acid (9:2:1) [15]. The quantitative estimation of total flavolignans there were carried out by the using of direct spectrophotometry [10].

2.4. The determination of antioxidant activity of substances

The examination of antioxidative actions of flavolignans 1 and **2**, and *Silybum marianum* fluid extracts are carried out on the rats (Wistar), at which under a narcosis the liver remove, and from it prepared a homogenate in a glass homogenizer at + 1 °C with the use of 0,05 M phosphatic buffer. In all series of experiments the homogenate was taken from one animal, is divided on five portions, which were utilized in five series of experiences till nine assays in everyone. In all experiments the concentration of a malonic dialdehyde (MDA) by means of the reaction with thiobarbituric acid (TBA) according to well-known method [16] was determined. To 0,5 ml of the liver homogenate 5 ml 20% phosphotungstic acid were added. The mixture left on a cold before formation large flakes, then centrifuged. Filtrate was separated and to the sediment added 2 ml of distilled water and 1 ml of the solution of TBA which prepared by dissolution of 80 мг TBA in 5 ml acetic acid. Samples in test tubes incubate in a boiling water bath at 99-100 °C. The test tubes cooled, centrifuged for 10 min at 8000 rpm, and then measured an optical density of the filtrates on a spectrophotometer at a wavelength 535 nm and 580 nm. Calculation of the concentration of MDA yielded according to expression reported in paper [16]:

$$C = 0,21 + 26,5\ \Delta D,$$

C – The concentration of MDA in a nMol/ml of the homogenate from the liver rats;

ΔD - optical density at a wavelength 535 nm minus optical density at a wavelength 580 nm.

Five series of experiments are carried out. In all investigated samples (excepting the first sample as control 1) there was make the boosted lipid peroxidation by means of addition of the well-known stimulator of this process - FeSO4 in terminating concentration 0,076 mg/ml (0,5 mM). At that the second sample was control 2. In the 3-rd and 4-th series of experiments was added silybin (1) and 2,3-dehydrosilybin (2) in the concentration of 2 mM/l. In the fifth sample was added *Silybum marianum* fluid extracts in such quantity, that the total content of flavolignans 1 and 2, contained in this preparation, was 2 mM/l in medium.

3. Results and Discussion

3.1. TLC-Analysis and the Quantitative Determination of Total Flavolignans

On the TLC-chromatograms of the *Silybum marianum* drugs and prepared fluid extract flavolignans 1 and 2 (yellow in the *vis*) were observed after the treatment with the diazobenzolsulfoacid reagent as the orange zones (Rf ~ 0,7 and Rf ~ 0,75 respectively) (solvent systems: chloroform-acetone-formic acid, 9:2:1). Both have the same meaning silydianin and silychristin (Rf ~ 0,55 and Rf ~ 0,4 respectively) on the TLC-chromatograms were detected. The quantitative estimation of total flavolignans by the using of direct spectrophotometry [10] was shown that the content of these compounds in the fluid extract is 2,75% (on the calculate of silybin).

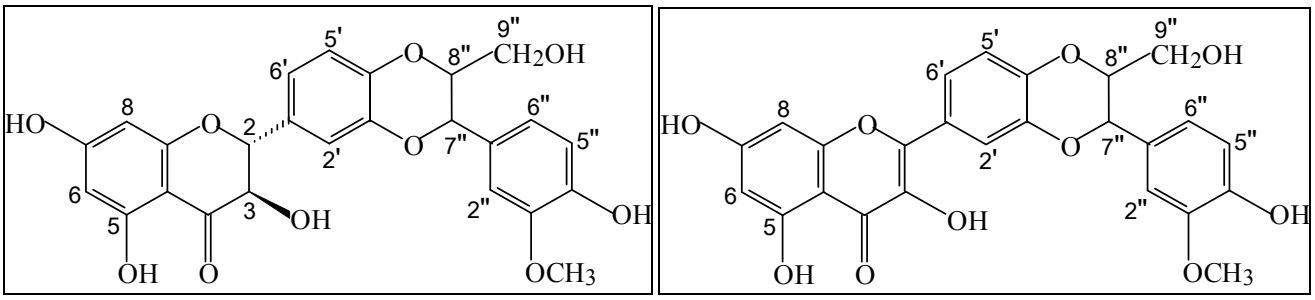
3.2. Physical-chemical, Spectral Characteristics of Isolated Compounds and Their Structural Elucidation

Silybin (**1**)

White crystals (acetone-EtOH); m.p. 164-166 °C; + 10° (c 0,05, acetone); $C_{25}H_{22}O_{10}$ (M^+ 482); UV spectra: λ_{max} nm EtOH 289, 325("shoulder"); ^{1}H-NMR spectra at 50 °C (300 MHz, $(CD_3)_2CO$) δ: 11,70 (1H, s, 5-OH), 6,8-7,2 (6H, m, H-2', H-5', H-6', H-2'', H-5'', H-6''), 5,96 (2H, *J*=d, 2 Hz, H-6,8), 5,10 (1H, *J*=d, 12 Hz, H-2), 5,00 (1H, *J*=d, 8 Hz, H-7''), 4,60 (1H, *J*=d, 12 Hz, H-3), 4,1-4,2 (1H, m, H-7''), 3,3-3,8 (1H, m, 2H-9''), 3,85 (3H, s, CH_3O). Compound (1) was identified as silybin [17].

2,3-Dehydrosilybin (**2**)

Yellow crystals (water-EtOH); m.p. 253-255 °C; $[\alpha]_D^{20} \pm 0°$ (c 0, 1, acetone); $C_{25}H_{20}O_{10}$ (M^+ 480); UV spectra: λ_{max} nm EtOH λ_{max} 267, 365 нм. ^{1}H-NMR spectra (300 MHz, $(CD_3)_2CO$) δ: 12,15 (1H, s, 5-OH), 9,74 (1H, br. s, 7-OH), 7,87 (1H, *J*=d, 2,1 Hz, H-2'), 7,17 (1H, *J*=dd, 8,3 and 2,1 Hz, H-6'), 7,8-6,9 (4H, m, H-5', H-2'', H-5'', H-6''), 6,60 (1H, *J*=d, 2,1 Hz, H-8), 6,28 (1H, *J*=d, 2,1 Hz, H-6), 5,06 (1H, *J*=d, 8,06 Hz, H-7''), 4,24 (1H, m, H-8''), 3,88 (3H, s, CH_3O), 3,80 (1H, *J*=dd, 12,5 and 3,5 Hz, H-9''), 3,55 (1H, *J*=dd, 12, 5 and 4,3 Hz, H-9''). Compound (2) was identified as 2,3-dehydrosilybin [12, 13].



Silybin (1) 2,3-Dehydrosilybin (2)

Fig. 1: Flavolignans from fruits of Silybum marianum (L.) Gaertn.

3.3. The Study of Antioxidant Activity of Substances

The rats were divided into 4 groups on 12 animals in each. The induction of the hepatotoxicity was carried out by means 50% oil solution of CCl4 which entered to the rats daily intramuscularly within 6 days to according to the method [8]. The doses of silybin (3-rd series), 2,3-dehydrosilybin (4-th series) and the fluid extract of Silybum *marianum* fruits **(**5-th series) which were injected to the animals are 25 mg/kg, 25 mg/kg and 150 mg/kg, respectively. There was established, that in the first series of experiments (control 1) (Table 1) the lipid peroxidation in the liver homogenate has made 2,80±0,064 nMol/ml of a homogenate. In the second series of experiments (control 2) there was determined the boosted level of lipid peroxidation (3,67±0,238 nMol/ml) induced by FeSO4 (Table 1).

Table 1: Influence of the substances from the fruits of Silybum marianum on the content of MDA (nMol/ml) in a liver homogenate

The content of MDA, nMol/ml				
1-st series	**2 series**	**3-rd series**	**4-th series**	**5-th series**
The liver homogenate (control 1)	The liver homogenate containing 0,5 mM $FeSO_4$ (control 2)	The liver homogenate containing 0,5 mM $FeSO_4$ + silybin (1)	The liver homogenate containing 0,5 μM $FeSO_4$ + 2,3-dehydrosilybin (2)	The liver homogenate containing 0,5 mM $FeSO_4$ + fluid extract
2,80±0,064	3,67±0,238	2,20±0,142 p <0,001	1,93±0,106 p <0,001	1,61±0,08 p <0,001

The results of the carried out examinations have shown, that both flavolignans, silybin (1) and 2,3-dehydrosilybin (2), decrease the level of the lipid peroxidation in the liver homogenate: concentration of MDA reduces with 3,67+0,238 nMol/ml up to 2,20+0,142 nMol/ml and 1,93+0,106 nMol/ml accordingly (Table 1). It is necessary to note, that the antioxidant activity of 2,3-dehydrosilybin (2) is reported for the first time and on 14,0% above those of silybin (1). More expressed antioxidative actions the effect in comparison with flavolignans 1 and 2 were marked for *Silybum marianum* fluid extract, which decreases a level of the lipid peroxidation in a homogenate (control 2) in 2,4 times (Table 1), and this background is much lower (in 1,7 times) than an initial test (control 1) (Table 1). Thus, silybin (1), 2,3-dehydrosilybin (2), and also new preparation, an fluid extract *Silybum marianum,* have expressed antioxidative actions, decreasing a level of the lipid peroxidation caused $FeSO_4$ in a homogenate from a liver rats.

4. Conclusions

The flavolignans silybin (1) and 2,3-dehydrosilybin (2) were isolated from fruits of milk thistle [*Silybum marianum* (L). Gaertn.] cultivated in the Samara region (Russian Federation). 2,3-dehydrosilybin (2) for the first time is described for the given plant, cultivated in Russian Federation. Silybin and 2, 3-dehydrosilybin and also the fluid extract of *Silybum marianum* fruits*,* have expressed antioxidant activity, decreasing the level of the lipid peroxidation caused $FeSO_4$ in the homogenate of a liver rats. The antioxidant activity of 2,3-dehydrosilybin is reported for the first time and on 14,0% above those of silybin. The discovery of the antioxidant activity for 2,3-dehydrosilybin allows in a new fashion to discuss the contribution of flavolignans in biological activity of hepatoprotective remedies on the basis of *Silybum marianum* drugs.

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