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Antioxidant Activity of Flavolignans from *Silybum marianum* (L.) Gaertn. Fruits

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Research Article

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ABSTRACT

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 in Russian Federation is described for the given plant. 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 FeSO₄ in the homogenate of a rat liver. 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. of flavolignans in biological activity of hepatoprotective remedies on the basis of *Silybum marianum* drugs.

INTRODUCTION

The flavolignans silybin (**1**) and 2,3-dehydrosilybin (**2**) (**Figure 1**) 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 in Russian Federation is described for the given plant. Silybin and 2,3-dehydrosilybin and also new preparation, a fluid extract of *Silybum marianum* fruits, have expressed antioxidant activity, decreasing the level of the lipid peroxidation caused FeSO₄ in the homogenate of a rat liver. 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.

The drugs of milk thistle [*Silybum marianum* (L.) Gaertn.] (Legalon, Carsil, Silybinin Silymar, fluid extract etc.)^[1-7] have ability to inhibit a lipid peroxidation in a tissue of a liver and to increase the activity such antioxidant enzymes, as superoxidismutase and catalase^[8]. The unique hepatoprotective activity of preparations from *Silybum marianum* fruits is caused by flavolignans, 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 flavolignans and namely silandrin, silyhermin and silymonin which are desoxyanalogues of silybin, silychristin and silydianin respectively^[11]. According to our results^[11] in the *Silybum marianum* fruits in appreciable amounts contains 2,3-dehydrosilybin (**2**) (**Figure 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 biological activity. Recently for silybin and 2,3-dehydrosilybin the free radical scavenging activity was described^[14].

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 ^[15].

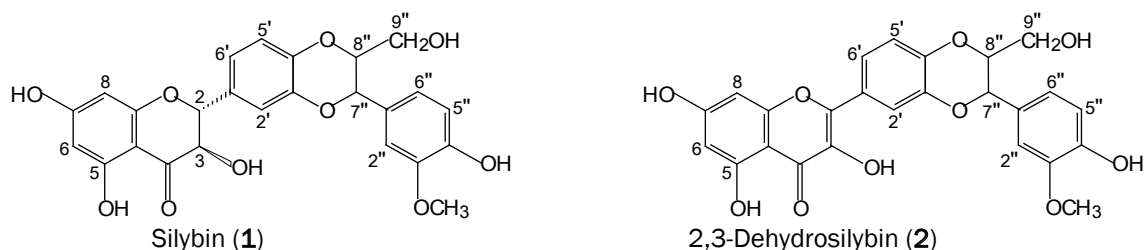


Figure 1. Flavolignans from fruits of *Silybum marianum* (L.) Gaertn.

MATERIALS AND METHODS

Extraction of Plant Materials

The isolation of silybin (**1**) 2,3-dehydrosilybin (**2**) (**Figure 1**) was carried out from the fruits of *Silybum marianum* (L.) Gaertn. cultivated in the Samara region (Russian Federation). A voucher specimen was deposited at the 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 (60 °C). 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 of on polyamide “Woelm” (8 x 20 cm) (solvent gradient systems: water/EtOH).

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 (4 x 20 cm) (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 ^[15] by means of extraction of thus drugs with 80% EtOH.

Methods of Structural Elucidation and Analysis of Plant Materials

¹H-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 was 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: CHCl₃-MeOH-H₂O (26:14:3) and chloroform-acetone-formic acid (9:2:1) ^[16]. The quantitative estimation of total flavolignans there was carried out by the using of direct spectrophotometry ^[10].

The Determination of Antioxidative Activity of Substances

The examination of antioxidative actions of flavolignans **1** and **2**, and *Silybum marianum* fluid extracts is carried out on the rat liver, 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 ^[17] 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 mg 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 ^[17]:

$$C = 0.21 + 26.5 \Delta D,$$

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.

RESULTS AND DISCUSSION

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 diazobenzenesulfonic acid reagent as the orange zones ($R_f \sim 0,7$ and $R_f \sim 0,75$ respectively) (solvent systems: chloroform – acetone-formic acid, 9:2:1). Besides also silydianin and silychristin ($R_f \sim 0.55$ and $R_f \sim 0.4$ 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).

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

3.2.1 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, 325sh; 1H -NMR spectra at 50 °C (300 MHz, $(CD_3)_2CO$) δ : 11.70 (s, 5-OH), 6.8-7.2 (m, 6H-Ar), 5.96 (d, 2H, 2 Hz, H-6,8), 5.10 (d, 12 Hz, H-2), 5.00 (d, 8 Hz, H-7¹¹), 4,60 (d, 12 Hz, H-3), 4.1-4.2 (m, H-7¹¹), 3.3-3.8 (m, 2H-9¹¹), 3.85 (s, 3H, CH_3O). Compound (1) was identified as silybin^[18].

3.2.2 2,3-Dehydrosilybin (2)

Yellow crystals (water-EtOH); m.p. 253-255 °C; $[\alpha]_D^{20} \pm 0^\circ$ (c 0.1, acetone); $C_{25}H_{20}O_{10}$ (M^+ 480); UV spectra: λ_{max} nm EtOH λ_{max} 267, 365 nm. 1H -NMR spectra (300 MHz, $(CD_3)_2CO$) δ : 12.15 (s, 5-OH), 9.74 (br. s, 7-OH), 7,87 (d, 2.1 Hz, H-2¹¹), 7,17 (dd, 8.3 and 2.1 Hz, H-6¹¹), 7.8-6.9 (m, 4H-Ar), 6.60 (d, 2.1 Hz, H-8), 6.28 (d, 2.1 Hz, H-6), 5,06 (d, 8,06 Hz, H-7¹¹), 4,24 (m, H-8¹¹), 3.88 (s, 3H, CH_3O), 3.80 (dd, 12.5 and 3.5 Hz, 1H-9¹¹), 3.55 (dd, 12.5 and 4.3 Hz, 1H-9¹¹). Compound (2) was identified as 2,3-dehydrosilybin^[12,13].

The Study of Antioxidative Activity of Substances

There was established, that in the first series of experiments (control 1) (**Table 1**) the lipid peroxidation in the rat 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 $FeSO_4$ (**Table 1**).

The results of the carried out examinations were 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. The determination of the antioxidant properties for 2,3-dehydrosilybin (**2**) allows in a new fashion to discuss the contribution of flavolignans in biological activity of hepatoprotective remedies on the basis of *Silybum marianum* drugs.

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

The content of MDA, nMol/ml				
1 st series	2 nd 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 mM $FeSO_4$ + 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 $p < 0,001$

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 in Russian Federation is described for the given plant. 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 the rat liver. 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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