MEDICINAL PLANTS

INTERACTION OF MILK-THISTLE-FRUIT FLAVANONOLS WITH LANGMUIR MONOLAYERS OF LECITHIN AND BILAYERS OF LIPOSOMES

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Interactions of silybin and dihydroquercetin (taxifolin), flavanonols from milk thistle [*Silybum marianum* (L.) Gaertn.] fruits, with Langmuir monolayers of lecithin and bilayers of liposomes are compared. It is established that the investigated flavanonols have different mechanisms of action. Taxifolin produces predominantly membrane-stabilizing action whereas silybin is characterized by immobilization in the hydrophobic part of the phospholipid bilayer with the formation of more hydrophilic micellar structures. The presence of silybin and taxifolin in the liquid extract of milk thistle fruits suggests that a dual mechanism of action may occur in this preparation and also in other combined medicines based on these fruits. The results of this study show good prospects for creating combined preparations based on milk thistle fruit, taxifolin, and other flavonoids (quercetin, rutin, diosmin, etc.) possessing pronounced membrane-stabilizing action.

Key words: milk thistle, *Silybum marianum* (L.) Gaertn., flavonoids, flavolignans, silybin, dihydroquercetin, lecithin, liposomes, membrane-stabilizing activity.

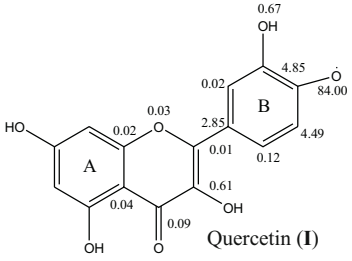
Biologically active compounds (BAC) of plant origin, in particular, flavolignans (phenylpropanoid class) and flavonoids biogenetically related to phenylpropanoids, are valuable components of phytopreparations and food additives [1 – 3]. Their action is based on strong antioxidant activity that can inhibit free-radical processes in cells at three different stages: initiation by binding of O_2. during the peroxide oxidation of lipids (POL) stage by reaction with both peroxides and lipid-peroxide radicals and during formation of the OH·-radical through chelation of Fe ions.

Many experimental studies in aqueous systems found the following structural elements of flavonoid molecules that are most important for antiradical activity:

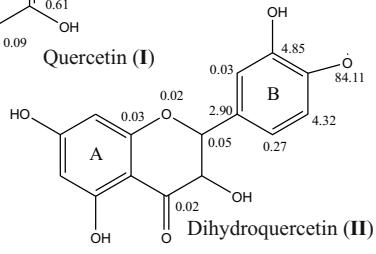
1. Two OH groups in the C-3[1] and C-4[1] positions.
2. A double bond between C atoms 2 and 3 that is conjugated to the C-4 carbonyl.
3. OH groups in the C-3 and C-5 positions in combination with a C-4 carbonyl.

Molecules of most flavonoids have two or all three structural groups that determine the antioxidant activity.

Structural analysis and experimental results are consistent with a direct relationship between the antioxidant activity of flavonols and the number of phenolic OH groups in their molecules. An OH group in the C-4[1] position in flavonols (**I**) and flavanonols (**II**) with five hydroxyls is the preferred target of radical attack.



Quercetin (**I**)



Dihydroquercetin (**II**)

The presence of an OH group on neighboring C atom C-3[1] (catechol structure) or C-3[1] and C-5[1] (gallic structure) facili-

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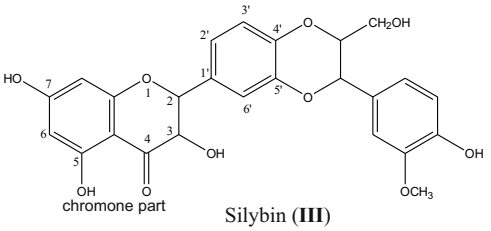
tates the loss of a H atom. H-bonds are formed between neighboring OH groups of ring B. Therefore, compounds with such structure typically have low oxidation potentials and form relatively easily radicals. Furthermore, the *ortho*-dihydroxyl structure results in extensive delocalization of the unpaired electron and increases the stability of the phenoxyl radical, as follows from the distribution of unpaired electron spin density about the quercetin and dihydroquercetin radical structures [4].

Silybin, being both a flavanonol and flavolignan [1, 2], has a very complicated chemical nature. The chromone part of the molecule exhibits weak acidic properties and can undergo donor-acceptor interactions with bases. The phenol hydroxyls are responsible for the interaction of silybin with strong bases and the dissolution in water as sodium (potassium) salts.



Silybin (**III**)

Silybin exhibits a high antioxidant capability owing to the polyphenol hydroxyls and the ability to form complexes with transition and other metal ions in the 3,4- or 4,5-positions.

The antioxidant properties of silybin are responsible for its high hepatoprotective, antitumor, and other effects [1, 2]. A comparison in the experimental system "CCl_4-induced POL in microsomes from rat liver" showed that the relative inhibiting or protective effectiveness of BAC decreases in the order luteolin > apigenin > datiscetin > morin > galangin > eriodictyol > (+)-catechin > gardenin D > silybin [5]. Furthermore, flavonoids of this group, silybin and silymarin (total flavolignans), are known to inhibit growth and DNA synthesis in various human tumor cell lines [6]. Silybin and dihydroquercetin reduced cholesterol synthesis in cells by inhibiting the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase [7]. The medical and biological activity of flavonoids has been thoroughly reviewed [8].

The effectiveness of flavonoid antioxidants in heterophase systems such as cells or lipoproteins is determined mainly by their lipophilicity and hydrophilicity. Flavonoids, like α-tocopherol and cholesterol, stabilize membranes and act as structural antioxidants. The flavonoid molecules reduce significantly the mobility of lipids, penetrating into the hydrophobic region of membranes. This in turn reduces the strength of the interaction of peroxide radicals with new lipid molecules.

$$RO_2^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

The hepatoprotective effect of silymarin and its main component silybin is due not only to the antioxidant activity

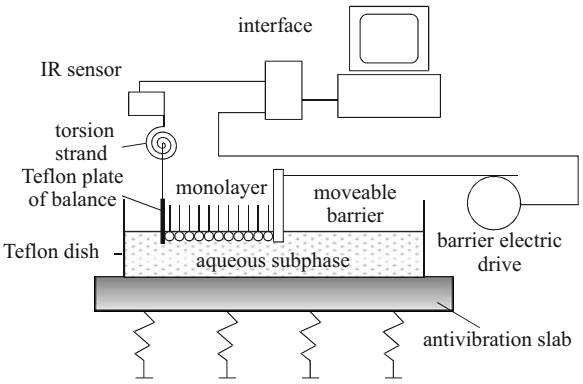


Fig. 1. Diagram of instrument for forming Langmuir films and determining compressibility parameters.

of these flavonoids but also to their membrane-stabilizing and metabolic activity (stimulation of protein biosynthesis, acceleration of regeneration of damaged hepatocytes) [9].

Thus, it can be seen that the pharmacological effects of flavonoids are determined to a large extent by their hydrophilic-lipophilic ratio and, therefore, their penetration through lipophilic bilayers of cell membranes.

The goal of our work was to study the interaction of silybin (**III**) and dihydroquercetin (**II**), the principal active components of phytopreparations based on extract of milk thistle, with Langmuir monolayers of lecithin and bilayers of liposomes as the simplest models of the lipid part of biomembranes. Information about the effect of these BAC on membranes allows not only the penetration and delivery pathway of drugs from preparations based on milk thistle, in which these compounds are found, to be predicted but also new effective drug forms of the preparation to be proposed.

EXPERIMENTAL PART

SSS silybin was prepared at the Pharmacognosy Faculty of Phytotherapy Botanicals of Samara State Medical University. Dihydroquercetin (98%) was obtained from ground larch wood (Bioxan grade) from OOO Rosbioprom (Sarov, Nizhegorod Oblast). Egg lecithin was prepared according to the literature method [10] and corresponded to 1-palmitoyl-2-oleyl-sn-glycero-3-phosphatidylcholine. Liquid Extract of Milk Thistle in ethanol (80%, 1:1, VFS-42-3381-99) was supplied by Samara Pharmaceutical Plant in collaboration with Samara State Medical University. The flavolignan content in this extract calculated for SSS silybin was 2.50%. Phytopreparation Marisil (TU 9146-002-14551985–04) was produced at Samaralektravy (Samara Oblast) and was the alcohol extract of milk thistle fruit (ZAO Samaralektravy manufacturer), namely, tincture in ethanol (80% 1:5) that did not contain adjuvants. The flavolignan content in Marisil phytopreparation calculated for SSS silybin was 0.53%.

Electronic absorption spectra of silybin and dihydroquercetin solutions, their mixtures, and solutions of milk

thistle phytopreparations in the range 200 – 500 nm were recorded on a Bioline Specord S 100 instrument (Analytik Jena AG) in 10-mm quartz cuvettes. The solvents were alcohol, CH_3CN, and universal buffer mixture. Flavonoids were identified by HPLC on a Shimadzu LC-10 chromatograph. SSS flavonoids were analyzed on a C_{18} column with gradient mobile phase CH_3CN:H_2O at flow rate 1 mL/min. Absorption spectra of silybin and dihydroquercetin have a band at 230 nm and a band with a maximum at 287 nm (silybin) and 290 nm (dihydroquercetin). HPLC chromatograms were compared by detection at wavelengths 287 and 290 nm for solutions of pure silybin and dihydroquercetin, respectively, and at 287 for the alcohol extracts. Elution was performed using the following regime:

τ, min	CH_3CN:H_2O volume ratio
0	95:5
10	5:95
15	5:95

1. Preparation of monomolecular film and compression isotherms

Monolayers were studied using a custom-built automated apparatus from the Physical Engineering Faculty of Nizhegorod State Technical University (Fig. 1). A Langmuir balance equipped with an IR sensor measured the surface pressure to an accuracy $\pi = 0.05$ mN/m. The uncertainty in the molecular area was at most 2 – 5%. The dimensions of the Teflon dish were 200 × 137 × 2 mm. The specific compression rate (per molecule) at these Langmuir dish sizes was about 1.2 Å^2/min · molecule. Monolayers were prepared using lecithin (1-palmitoyl-2-oleyl-sn-phosphatidylcholine) as the simplest model of the lipid part of the membrane and its mixtures with silybin and dihydroquercetin. The spreading time and compression conditions were selected so that the compression isotherms did not have hysteresis. Doubly distilled water was used as the subphase.

Preparations for the experiment included cleaning of the Teflon dish and the stationary and movable barriers to the chromone mixture and doubly distilled water. Then, the Teflon dish was filled with the subphase and the barriers were set. A solution of the studied compound was placed on the subphase surface. The time to reach full equilibrium after evaporation of solvent was 30 min. Measurements were made 30 min after the film had completely spread over the subphase surface.

2. Liposome preparation

Silybin or dihydroquercetin or their mixture (1 mg) was dissolved in ethanol (2 mL) with heating on a water bath. The membrane-forming component (lecithin, 36 mg) was dissolved in $CHCl_3$ (8 mL). The resulting mixture was evaporated to remove organic solvent and dried in a round-bottomed flask under reduced pressure (water aspirator) to form a film. The resulting film under a stream of Ar was treated with doubly distilled water (50 mL) as the dispersed phase. The contents were stirred until the film was completely rinsed. The resulting suspension was processed in a UZDN-2T ultrasonic bath at 44 kHz for 1 – 2 min. The dry extract obtained by distillation of solvent from the alcohol extracts was used to prepare liposomes with phytopreparations. Unincorporated material was separated by gel filtration over columns of Sephadex G-25 or G-50 (total volume 44.0 mL). Liposomes obtained after gel filtration were passed through a column of Sepharose 4 B-CL (column volume 45.2 mL) at 4°C.

Incorporation of flavonoids into liposomes was monitored before and after filtration by electronic spectroscopy using the transmission coefficient (*T*, %) of an aqueous liposome suspension in the visible region and by HPLC with detection at 287 nm.

The dimensions of the vesicles were estimated by atomic-force microscopy (AFM) in a Solver Bio NT-MDT (Zelenograd) scanning microscope in noncontact mode. The liposome suspension was placed on the glass substrate (1 mm thickness). The dispersion medium was removed by vacuum drying.

RESULTS AND DISCUSSION

UV spectra of alcohol preparations Liquid Extract of Milk Thistle and Marisil typically had strong absorption at 190 – 210 nm (absorption of polyphenol fragments, usually phenol ring A in the chromone part of the flavonoids) and a main band at λ_{max} 288.89 nm that was broadened by a shoulder at $\lambda = 325.03$ nm (Fig. 2*a*). The main absorption band corresponded to absorption of the chromone fragment of dihydroquercetin with $\lambda_{max} = 290.12$ nm and silybin with $\lambda_{max} = 288.49$ nm (Fig. 2*b* and 2c, respectively). The alcohol solution of the dihydroquercetin and silybin mixture (SSS samples) gave an additive UV spectrum (Fig. 2*d*).

These BAC could be separated using HPLC with detection at 287 nm, a C_{18} column, and mobile phase CH_3CN:H_2O at 40°C. The relative quantitative characteristics of Liquid Extract of Milk Thistle and Marisil were obtained (Fig. 3, a-d).

TABLE 1 lists the calculated mass contents (%) of silybin and dihydroquercetin.

HPLC analysis showed that the mass content (%) of dihydroquercetin in Liquid Extract of Milk Thistle was almost 1.7 – 1.8 times less than in phytopreparation Marisil for the same concentration of main active component (silybin) (Table 1).

Considering that dihydroquercetin not only is a broad-spectrum antioxidant but also exhibits P-vitamin activity [3], a change of the dihydroquercetrin-silybin mass ratio at constant silybin concentration can probably change the mechanism of hepatoprotective activity, including the delivery pathway of the active component to the biological target and its penetration through lipid fragments of biomembranes and/or their stabilization.

Two model systems were used to study the influence of silybin and dihydroquercetin on the lipid fragment of

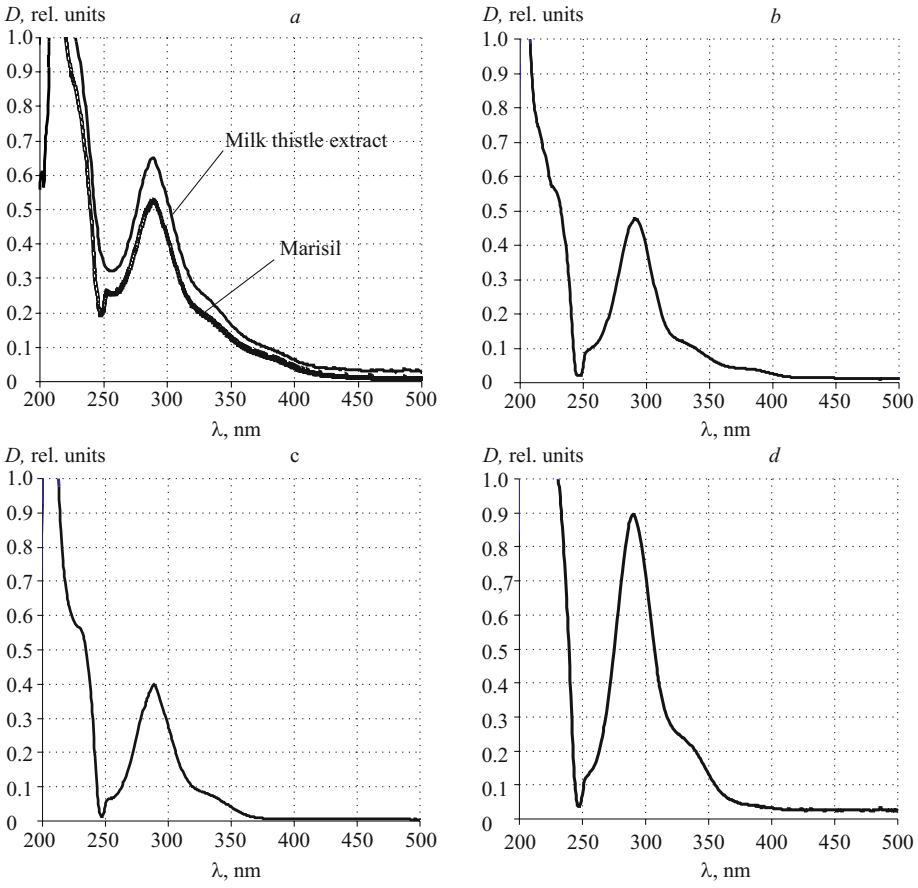


Fig. 2. Electronic absorption spectra of alcohol solutions of Marisil and Liquid Extract of Milk Thistle (*a*), silybin (5×10^{-5} M) (*b*), dihydroquercetin (5×10^{-5} M) (*c*), mixture containing silybin (5×10^{-5} M) and dihydroquercetin (5×10^{-5} M) (*d*).

biomembranes. The membranes in the first model system were Langmuir monolayers formed by amphiphilic compounds on the surface of the aqueous solution (subphase) [11]. The Langmuir monolayer can consist of only lipids (homogeneous surface film) or a mixture of lipids and other BAC (mixed film). The subphase can be any polar liquid including biological media, blood plasma, aqueous solutions of drugs and inorganic salts, isotonic solutions, water, etc., on the surface of which a stable monomolecular film can be produced.

The second methodological approach to studying the interaction of drugs with biomembranes was based on the use of phospholipid vesicules or liposomes [12]. Vesicules included hollow spherical nanoparticles with a closed shell of a lipid membrane (mono- or multi-layer), inside of which the aqueous solution was encapsulated.

We used 1-palmitoyl-2-oleyl-sn-glycero-3-phosphatidylcholine (lecithin). The formula of lecithin shows (Fig. 4) that this compound contains both ionizable and nonpolar groups, i.e., is amphiphilic. Therefore, studied BAC with amphiphilic properties were also included in the phospholipid membrane.

A known amount of lipid or its mixture with BAC dissolved in a volatile solvent ($CHCl_3$ or C_2H_5OH:$CHCl_3$ mix-

TABLE 1. Content of Dihydroquercetin and Silybin in Milk-Thistle Phytopreparations (HPLC)

Fig. 3	Medicinal agent	Concentration of active compounds, g/100 mL	Retention time, τ, min/*E*, mV		Content in extract, %	
			silybin	dihydroquercetin	silybin	dihydroquercetin
c	Liquid Extract of Milk Thistle	0.014	9.115/180	7.729/15	10.71	4.29
d	Marisil	0.0092	9.104/105	7.709/20	10.87	7.61
a	SSS silybin	0.0096	9.079/700	–	99.98	–
b	SSS dihydroquercetin	0.0092	–	7.692/240	–	99.22

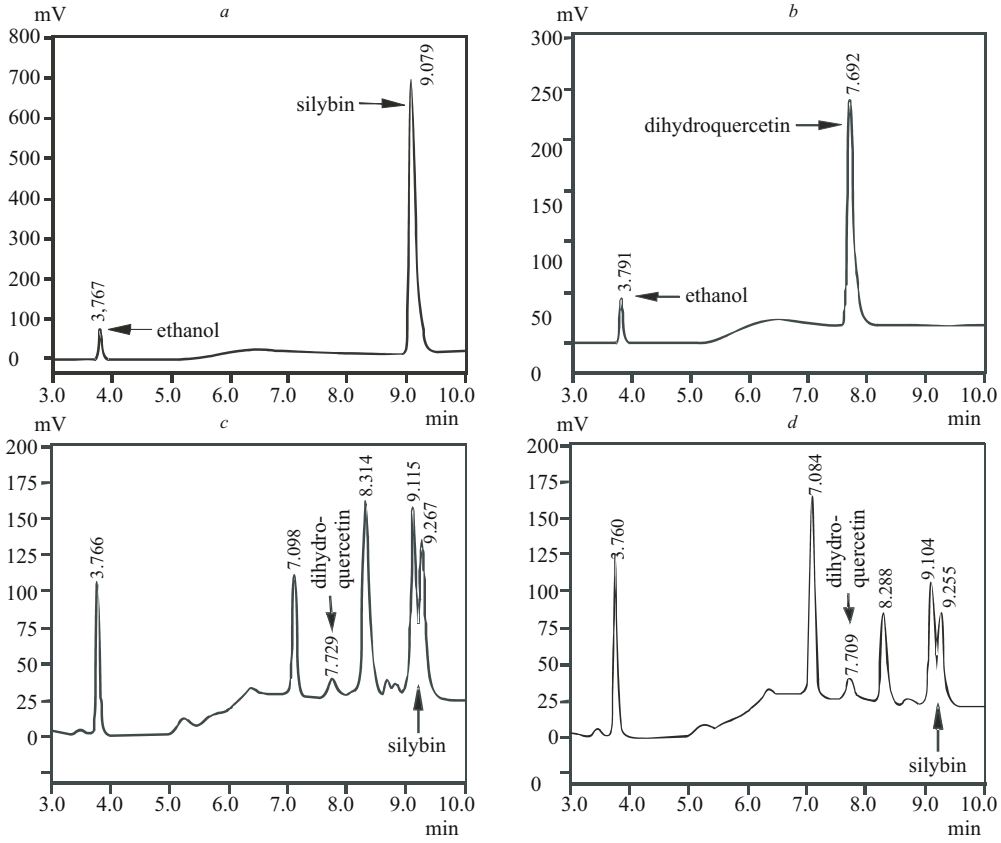


Fig. 3. HPLC of alcohol solutions of SSS silybin (a), SSS dihydroquercetin (b), phytopreparation Liquid Extract of Milk Thistle (c), and phytopreparation Marisil (d).

ture) was placed on the aqueous surface to form the insoluble monolayer. The surface area S and surface pressure $\pi = \gamma_0 - \gamma$, where γ and γ_0 are the surface tension of the aqueous solution with the applied monolayer and without it, were measured after solvent was completely removed by compressing the monolayer.

Figures 5 – 7 show isotherms of surface potential $\pi = f(S_0)$ for a monolayer of egg lecithin and its mixtures with BAC. The quantity S_0 was characterized as the area occupied by one lecithin molecule in a closest packed monolayer that modeled a biomembrane.

The compression isotherm $\pi = f(S_0)$ for the lecithin monolayer had a form typical for compounds of this type (Fig. 5).

Extrapolation to $\pi = 0$ enables the molecular area of lecithin $S_0 = 0.55$ nm$_2$ to be estimated. The theoretically calculated area occupied by two closest packed hydrophobic lecithin chains is $S_0 = 0.41$ nm$_2$. However, the lecithin molecule under actual conditions in the monolayer contains adsorbed water in its polar part so that $S_0 = 0.52 - 0.56$ nm$_2$.

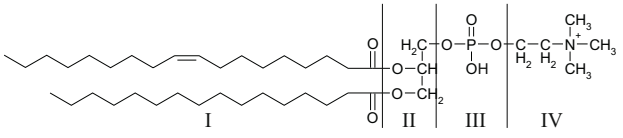


Fig. 4. Structure of lecithin: fatty-acid moieties (I), glycerine moiety (II), phosphate group (III), choline part (IV).

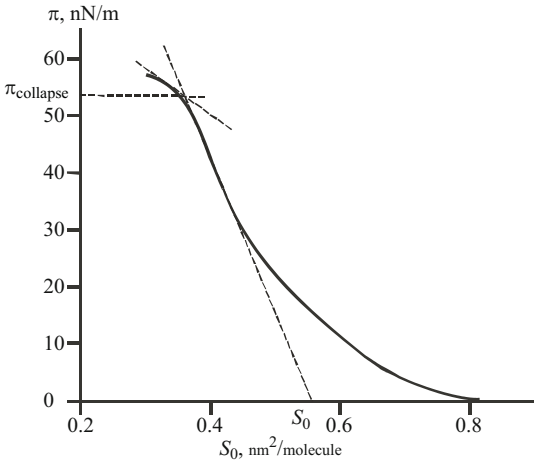


Fig. 5. Compression isotherm $\pi = f(S_0)$ of lecithin monolayer on surface of doubly distilled water (subphase), $t = 26$°C, $C = 3.9 \times 10^{-8}$ mol/subphase surface, $V = 30$ μL.

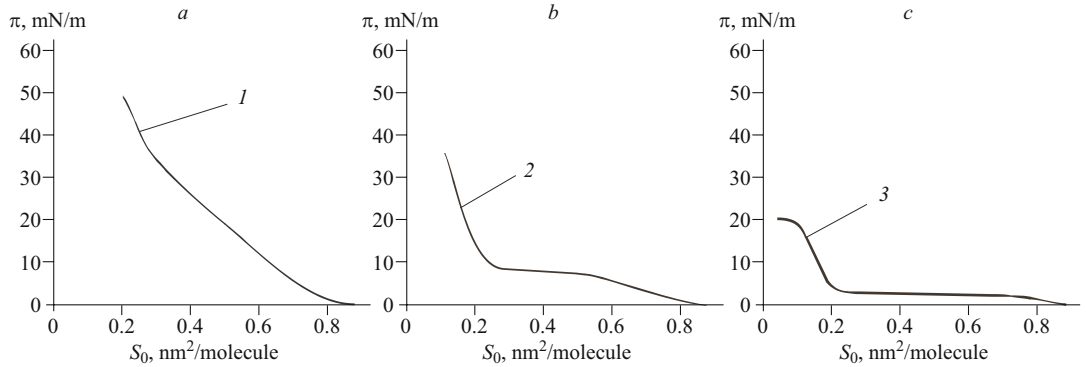


Fig. 6. Compression isotherms $\pi = f(S_0)$ of licithin-silybin system (15:1). Lecithin concentration 5.25×10^{-8} mol/subphase surface; silybin, 3.46×10^{-9} mol/subphase surface; $t = 26$°C.

The surface pressure of collapse $\pi_{collapse}$ for lecithin monolayers depends on the nature of the lipid and lies in the range $\pi_{collapse} = 50 – 60$ mN/m. Figure 5 shows the compression isotherm of the lecithin monolayer, which is consistent with formation of a true monolayer because hysteresis (different compression and expansion curves) is not seen.

The compression isotherms of lecithin-silybin mixtures (Fig. 6, a – c) were fundamentally different. Two regions differing in the compressibility parameter $\beta = d\pi/dS$ could be seen on the $\pi = f(S_0)$ curves. The extrapolated values S_0 ($\pi = 0$) for a freshly prepared mixture were 0.83 – 0.87 nm^2 and 0.48 – 0.52, in regions **I** and **II**, respectively (Fig. 6*a*, curve 1). The surface pressure of collapse of the mixed layer decreased to 50 mN/m. The change of effective molecular area of lecithin in the presence of silybin in the mixed layer suggests that the monolayer components experienced intermolecular interactions. The interaction between silybin and lecithin probably occurred during preparation of the solution for depositing the monolayer after mixing the $CHCl_3$ solution of lecithin and the alcohol solution of silybin. This hypothesis was confirmed by the data shown in Fig. 6*b* and 6c (curves 2 and 3). The effective molecular area of lecithin was practically halved from 0.54 ± 0.04 to 0.23 ± 0.03 nm$_2$ after storage for 4.5 h of the mixed $CHCl_3$:alcohol solution containing lecithin and silybin. The surface pressure of collapse decreased sharply from 55 ± 5 to 18 ± 5 mN/m. Furthermore, The intermolecular interaction between the components in solution affected the condition of the mixed layer. Horizontal portions with $\pi = \text{const}$ that were typical of a two-dimensional surface transition from one layer structural state to another were clearly visible on the compression isotherms $\pi = f(S_0)$ (2) and (3).

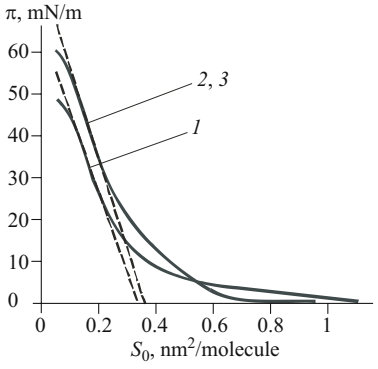


Fig. 7. Compression isotherms of lecithin-dihydroquercetin mixed film. Lecithin-dihydroquercetin molar ratios 1:1, lecithin concentration 4.72×10^{-8} mol/subphase surface; dihydroquercetin, 4.7×10^{-8} mol/subphase surface; $t = 25$°C (1); 10:1, lecithin concentration 4.1×10^{-8} mol/subphase surface; dihydroquercetin, 4.3×10^{-9} mol/subphase surface; $t = 23$°C (2); 15:1, lecithin concentration 4.1×10^{-8} mol/subphase surface; dihydroquercetin, 6.45×10^{-9} mol/subphase surface; $t = 23$°C.

The shape of the isotherms and the compression parameters ($S_0 = 0.23 \pm 0.03$) in the mixed layer that was prepared from the aged solution (stored for 4.5 h) were reasonable for a surface structuring and micelle formation. Therefore, the reaction mixture of silybin and lecithin even at a 1:15 mole ratio can lead to the formation of a compact bilayered structure or surface micelles with the polar part interacting more strongly with water. The quantity π, which reflects the intermolecular interaction between the layer components and the water molecules decreased from 50 – 60 to 18 mN/m. This agreed well with the aforementioned hypotheses (Table 2).

Dihydroquercetin had a smaller effect on the condition of the lecithin monolayer than silybin (Fig. 7).

The condition of the mixed monolayers did not depend on the storage time of the solution from which the monolayer was formed (from 1 min to 5 h). Changing the lecithin: dihydroquercetin molar ratio in the mixed layer from 15:1 to 1:1 did not substantially affect the shape of the isotherms but did change significantly the effective molecular area from 0.54 ± 0.02 to 0.35 ± 0.02 nm^2.

The surface pressure in the collapse region was reduced from 55 to 45 mN/m only for a 1:1 lecithin:dihydroquercetin ratio (Fig. 7). The most probable explanation for these facts is the immobilization of dihydroquercetin in the monolayer

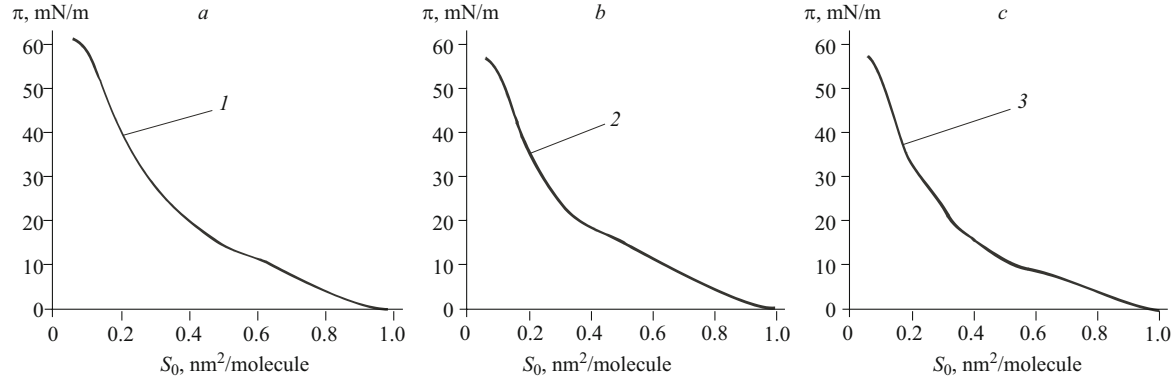


Fig. 8. Compression isotherms $\pi = f(S_0)$ of lecithin-Marisil mixed monolayers. Lecithin concentration 4.6×10^{-8} mol/subphase surface; silybin concentration 1.14×10^{-9} mol/subphase surface; t = 25°C. Storage time (τ) = 1 min (*1*), 2.5 h (*2*), 4.5 h (*3*).

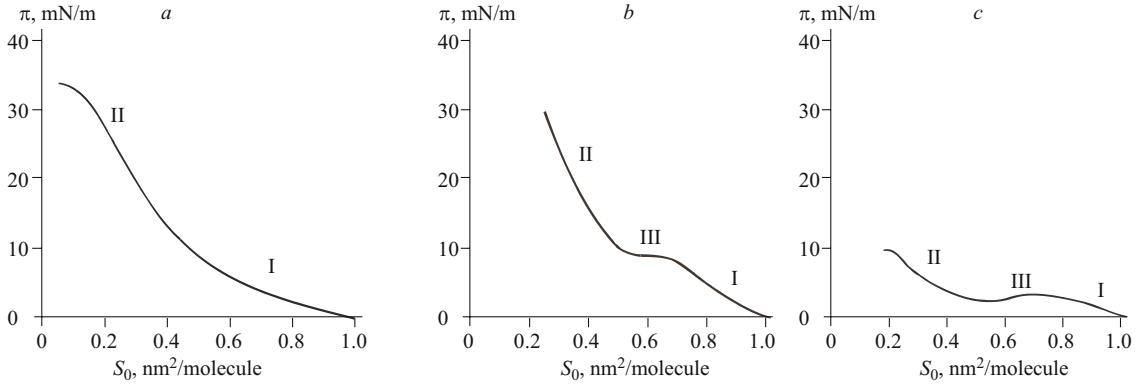


Fig. 9. Compression isotherms $\pi = f(S_0)$ of lecithin-milk-thistle-extract system. Lecithin concentration 4.6×10^{-8} mol/subphase surface; silybin, 1.5×10^{-9} mol/subphase surface: freshly prepared mixture (*a*), mixture stored for 4.5 h (*b*), mixture stored for 7.5 h.

together with the formation of a surface layer of a different structure than the single-component lecithin layer. Because the monolayer in general remains lipophilic ($\pi_{collapse}$ is large), it can be assumed that dihydroquercetin is adsorbed on the monolayer surface (monolayer-air interface) due to the penetration and permeation of dihydroquercetin through the hydrophobic part of the lecithin or mixed monolayer. It should be noted that dihydroquercetin stabilized the mixed monolayers with lecithin.

The important points for forming liposomes of phytopreparations containing the studied flavonoids are the nature of their inclusion from the alcohol extracts into the lipid layers and the stabilization or destabilization of the lipid layers in the presence of the flavonoids.

It was found that stable mixed monolayers of lecithin and the BAC obtained from the alcohol extracts could be obtained only with a large excess of lecithin in the monolayer, e.g., the lecithin:silybin (from Marisil extract) ratio was 40:1 (Fig. 8) whereas the lecithin:silybin ratio (from Liquid Extract of Milk Thistle) was 30:1 (Fig. 9). It is noteworthy that the same quantity of the preparations ($4.6 \cdot 10^{-4}$ g of alcohol extracts) was placed on the subphase surface. A comparison of Figs. 8 and 9 shows that mixed layers from the lecithin:Marisil extract system (Fig. 8), in which the absolute amount of silybin was less whereas the surface concentration

TABLE 2. Properties of Mixed Monolayers of Lecithin and Silybin as a Function of Storage Time of Nonaqueous Solutions Before Monolayer Formation

Isotherm	System	$\pi_{collapse}$, mN/m	$\pi_{phase\ transition}$, mN/m	S_0, nm²/molecule	S_1, nm²/molecule
1	Freshly prepared mixture	50 ± 3	–	0.50 ± 0.02	0.85 ± 0.02
2	Mixture stored for 1.5 h	35 ± 2	6 – 7	0.26 ± 0.03	0.85 ± 0.02
3	Mixture stored for 4.5 h	18 ± 5	3	0.23 ± 0.03	0.87 ± 0.03

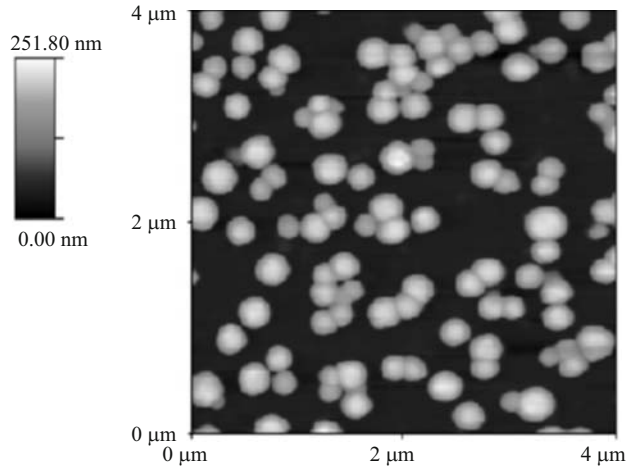


Fig. 10. AFM visualization of liposome suspension prepared from Liquid Extract of Milk Thistle.

of dihydroquercetin was greater than in mixed layers formed from the lecithin:Liquid Extract of Milk Thistle system (Fig. 9), were more stable for the same amount of alcohol extracts but different amounts of silybin in the Marisil and Liquid Extract of Milk Thistle preparations on the subphase surface. The results confirmed our observations that silybin possesses more solubilizing capability than dihydroquercetin and has a greater capacity to form surface associates with lecithin that decrease greatly the monolayer-water interphase tension. In general, both the silybin:dihydroquercetin ratio and the amount of dry BAC in the extracts affect the stability of the mixed monolayers prepared from the lecithin-alcohol extracts system (Table 1).

An analysis of compression isotherms of multicomponent layers formed by Marisil and lecithin (Fig. 8 and Table 3) indicates that the effective molecular area of lecithin S_0 and $\pi_{collapse}$ in the mixed multicomponent layer are close to S_0 in the two-component lecithin-dihydroquercetin layer.

The decrease of mass fraction of dihydroquercetin and the silybin:dihydroquercetin mass ratio by 1.7 – 1.8 times in Liquid Extract of Milk Thistle compared with Marisil destabilized the lecithin-extract of milk thistle mixed layer.

Surface compression isotherms of mixed monolayers $\pi = f(S_0)$ had several two-dimensional states from the liquid film state (**I**), solid (or liquid-solid) state **II**, and two-dimensional transitions from one state in the layer into other states **III** (Fig. 9).

Considering that the effect of dihydroquercetin on the condition of mixed layers is less significant, the basic differences in the compression isotherms of the layers can be attributed to the effect of silybin. Dihydroquercetin, being a component of the mixed layer, helps to preserve the layers during compression in the condensed condition that typically is very rigid. Thus, the region of liquid layers with lower compressibility is insignificant. The effect of silybin is evident not only in the sharp decrease of molecular area and the reduction of $\pi_{collapse}$ to 0.1 – 2 mN/m in the liquid laeyr region but also in the appearance of an extended plateau in the liquid layer region.

Thus, it can be hypothesized that an increased fraction of silybin in the extract causes a reorganization of the surface membrane, for example, formation of surface micelles and disintegration of the lecithin monolayer.

The interaction of flavononols in milk thistle fruit with lipid layers was also studied using liposomes, in which the membrane-forming component was egg lecithin, which is based on 1-palmitoyl-2-oleyl-sn-glycero-3-phospha- tidylcholine, the compound from which the Langmuir monolayers were formed.

Figure 10 shows an AFM photograph of a suspension of liposomes including components of Liquid Extract of Milk Thistle preparation.

Analogous pictures were observed for liposome suspensions prepared from Marisil preparation and aqueous alcohol solutions of flavonoids dihydroquercetin and silybin. The average liposome particle size was 0.1 – 0.3 μm.

The inclusion of BAC into liposomes was estimated using visible electronic spectroscopy of a suspension and starting solutions from which the liposomes were formed in addition to HPLC analysis of the filtrate (Table 4).

A decrease of transmission coefficient T (%, turbidity is a function of size) in the electronic spectra is one piece of evidence that the flavonoids permeated the bilayer membrane.

An analysis of electronic trasmission spectra and HPLC analysis of the aqueous phase of a suspension of liposomes prepared from the lecithin-flavonoid mixed layer and the layer prepared from the studied phytopreparations after filtration of the suspension showed that the flavonoids had disappeared almost completely from the aqueous phase (Table 4). Liposomes prepared from the lecithin-silybin mixed layer were unstable (< 1 d). Adding an equimolar (to silybin) amount of dihydroquercetin to the mixed flavonoid-lecithin layer increased the stability of the liposomes (2 d). Con-

TABLE 3. Properties of Mixed Monolayers of Lecithin and Marisil as a Function of Storage Time of Nonaqueous Solutions Before Monolayer Formation

Isotherm	System	$\pi_{collapse}$, mN/m	S_0, nm^2/molecule
1	Freshly prepared mixture	58	0.41 ± 0.02
2	Mixture stored for 2.5 h	55	0.40 ± 0.02
3	Mixture stored for 4.5 h	53	0.35 ± 0.03

TABLE 4. Properties of Liposome Dispersion

Liposome content	*T*, (after 1 h) %		Flavonoid concentration, mg/100 mL			
	333 nm	416 nm	$C_{init.}$	$C_{after\ filtration}$		$C_{in\ water*}$
				τ = 3 min	τ = d	
Water	45.6	63.9	–	–	–	–
Dihydroquercetin	0.6	0.6	9.0	0.2	0.7	150.0
Silybin	0.5	0.4	7.2	0.05	1.5	9.6
Marisil	0.5	0.4	8.2	0.15	2.1	–
Liquid Extract of Milk Thistle	0.5	0.4	10.3	0.05	3.2	–

* Limiting concentration of flavonoids upon dissolution in water with ultrasound action.

sidering the exceedingly low solubility of silybin in water (Table 4) and the insignificant concentration of unincorporated silybin in the solution after filtration of the liposome suspension, it can be assumed that silybin was incorporated into the hydrophobic part of the phospholipid bilayer and formed micellar structures. The silybin molecules were most probably located within the membrane lipid layer through hydrophobic binding or adsorption on the liposome surface.

The lower stability of liposomes with silybin was probably due to a spontaneous transition of the micellar lecithin structures with silybin from the viscous state into discreet micelles. This hypothesis was confirmed by HPLC analysis, according to which the concentration of released silybin after storage of the liposome suspension for 1 d increased from 0.05 to 1.5 mg/100 mL.

The water solubility of dihydroquercetin is much greater than that of silybin. It can be assumed that dihydroquercetin will be located in both the membrane lipophilic bilayer and the aqueous phase (contents) of the liposomes (Table 4). The concentration of dihydroquercetin released from the liposomes in 1 d was less than that of released silybin (Table 4). This confirmed the role of dihydroquercetin in membrane stabilization and agreed with experimental results of the effect of dihydroquercetin on Langmuir monolayers. The large fraction of dihydroquercetin in Marisil preparation was probably explained by the higher stability of liposomes containing this preparation than that of liposomes containing Liquid Extract of Milk Thistle (Table 4).

It must be noted that the studied phytopreparations contained the main flavonoids silybin and dihydroquercetin and other BAC including silybin structural isomers. It has been shown [13] that flavonoids and isoflavonoids interact with lipid (cholesterol, α-tocopherol) bilayer membranes by the same mechanism. Therefore, it can be assumed that the effect of silybin and its structural isomers on the lecithin membrane will be the same.

Thus, our results show that the mechanisms of action for phytopreparations Marisil and Liquid Extract of Milk Thistle containing the same mass fraction of the main active compound silybin and having practically the same pharmacological (hepatoprotective) activity can differ substantially. A study of the interaction of silybin and dihydroquercetin with Langmuir monolayers of lecithin and bilayers of liposomes showed that these flavonoids have different mechanisms of action. Dihydroquercetin mainly stabilizes the membrane whereas silybin is immobilized in the hydrophobic part of the phospholipid bilayer and forms more hydrophilic micellar structures. Therefore, the presence of silybin and dihydroquercetin in Marisil and Liquid Extract of Milk Thistle preparations suggests that a combined mechanism of action may occur in these preparations and in other total drugs based on milk thistle fruit. The experimental results enable the potential of creating total and combined preparations based on milk thistle fruit with pronounced membrane-stabilizing action (quercetin, rutin, diosmin, etc.) to be assessed.

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