Treatment of animals with fatty liver disease using a drug based on the seeds of *Silybum marianum*


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Introduction

Medicinal plants are a source of various therapeutic preparations. Therefore, the aim of our work was to prepare a liposomal drug from extract of the seeds of *Silybum marianum* (L.) Gaertn., adding tocophorol acetate, lecithin, squalene, and Tween 80. The drug was used on the laboratory animals (rats) intramuscularly to measure the efficacy of treatment of experimentally modeled toxic fatty liver disease. The fatty infiltration of the liver in the rats was caused by tetrachloromethane (CCl₄). The efficacy of the liposomal drug based on the extract from *S. marianum* seeds was studied on 25 animals in which the liver pathology had been caused by 50% oil solution of CCl₄ administered in the dose of 5 ml per kg of body weight. The diseased rats were divided into five groups, each consisting five animals. Animals of the four experimental groups – first, second, third, and fourth - received the drug intramuscularly in the doses of 0.05, 0.25, 0.50, and 1.50 ml/kg of body weight three times every two days, respectively. At the same time, the control rats received three-time intramuscular injection of physiological solution in the dose of 0.5 ml/kg of body weight. Treatment of the animals with fatty liver disease by injections of the drug based on the extract from *S. marianum* seeds normalized the general condition, significantly improved the functions and structure of the liver. Biochemical studies of blood serum of the sick animals after the treatment revealed increased in albumin content, which may suggest reduction of the protein-synthesizing function of the liver. The normalization of the bile-forming and bile-excreting functions of the liver, and also elimination of cholestasis were evidenced by reduced contents of bile acids and total bilirubin and increased total cholesterol in the blood serum of the rats. After treating the animals with the created drug, we saw decrease in the activity of the liver-indicator enzymes (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, and glutamate dehydrogenase) in the blood serum, which is a sign of recovery of the structure of hepatocytes and elimination of cytolysis. Histological studies of the liver of the treated rats confirmed the positive effect of the liposomal drug on the organ’s structure. In the future studies, we plan to test this combination of agents in treatment of agricultural and domestic animals with liver pathologies.

Keywords: rats; tetrachloromethane; *Silybum marianum*; liposomal drug; treatment.
silymarin and other biologically active compounds of *S. marianum* were exerted on the structure of hepatocytes and the course of physiologically-biochemical processes in them (Zhihui et al., 2018; Khazaei et al., 2022). *Silybum marianum* has important antioxidant properties. Taking into account that the development of a liver pathology is often associated with oxidative stress, the high efficacy of silymarin for treatment of patients with various hepatopathies is first of all attributed to its high antioxidant potential (Javed et al., 2018; Martynyak et al., 2022). The antioxidant action of silymarin and other biologically active compounds in *S. marianum* L. is explained by their ability to neutralize free radicals and inhibit the lipid-peroxidation processes. At the same time, inflammatory processes in the liver are alleviated, and the membrane structures in damaged hepatocytes undergo intensive recovery (Juriáňová et al., 2018). The membrane-stabilizing action of *S. marianum*-based drugs promoted recovery of the activity of liver-indicator enzymes in the blood and normalized the main functions of hepatocytes (Tajnohmarandi et al., 2018; Bashchenko et al., 2020). At the same time, increases were observed in the transport of nutrients and biologically active compounds through the cellular membranes, in particular in activation in the export of bile salts (Adetuyi et al., 2021).

Therefore, extracts from the seeds of *S. marianum* are natural, ecological, multi-functional, and multi-purpose drugs. From the economic standpoint, their usage is practical because of the low price and prominent treatment effect, and also absence of side-effects on the organism (Abenavoli et al., 2018).

However, there are studies indicating that drugs made of *S. marianum*, when administered orally, have low bioavailability for most species of animals. This is explained by the fact that silymarin contains flavonolignans, which are insufficiently metabolized in the organism after per os administration due to fast conjugation in the cells of intestines and liver, and also its elimination into bile (Tvrdý et al., 2021). At the same time, extracts from the seeds of *S. marianum* are poorly soluble in water, which may hinder their metabolism (Adetuyi et al., 2021).

The objectives of the study were preparing a drug from extracts from *S. marianum* seeds, with addition of tocopherol acetate, lecithin, squalene; its conversion into a liposomal emulsion, prepared based on Tween 80; and its intramuscular injection to laboratory animals with experimentally modeled fatty liver disease in order to measure its treatment potential.

### Materials and methods

Maintenance, feeding, care, and all the procedures with the animals were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and the General Ethical Principles of Experiments on Animals, adopted by the First National Congress of Bioethics (Kyiv, 2001). The experiments were performed adhering to all the principles of humaneness, as stipulated in the Directive of the European Community.

For the study, we selected 31 clinically healthy male Wistar rats, aged three months, with 180–200 g body weight, kept in the standard vivarium conditions on the generally accepted diet. Of 31 animals taken for the study, three animals received sunflower oil intragastrically, through a tube, in the amount of 5 mL/kg of body weight, and the other 28 were subjected to modeling of the hepatic pathology by tetrachloromethane (*CCl*$_4$) intoxication. For this purpose, each rat was singly, through the tube, was administered 50% oil solution of *CCl*$_4$ in the dose of 5 mL per kg of body weight. To study the effect of tetrachloromethane on the functional condition and structure of the liver, every two days, we performed decapsulation of three animals that received sunflower oil and three animals that received *CCl*$_4$.

The next stages of the research were preparing a drug based on the seeds of *S. marianum* and studying its medical efficiency on rats with experimentally modeled toxic fatty liver disease (toxic fatty infiltration of the liver).

The drug we designed was prepared in the form of liposomal emulsion and contained 8.0 mL of aqueous and 1.5 mL of oil extracts from the fruits (seeds) of *S. marianum*. To the extracts of *S. marianum*, we added 0.15 mg of alpha-tocopherol acetate, 0.05 mL of squalene, 0.3 mL of lecithin, and also 0.04 mL of Tween 80, which, being a surfactant, promoted formation of a liposomal emulsion. A mixture of the said compounds was prepared and dispersed on an UZDN-1 ultrasound device at 22 kHz frequency for 2–3 min until formation of a homogenous emulsion.

The developed drug was tested on 15 animals with *CCl*$_4$-induced liver pathology. The sick rats were divided into five groups, five in each. The first group (control, with no treatment) received intragastric administration of physiological solution in the dose of 0.5 ml, per kg of body weight. The second, third, fourth, and fifth (experimental, received treatment) were intramuscularly injected the drug we developed in the doses of 0.05, 0.25, 0.50, 1.50 mL per one kg of body weight. Treatment of the ill animals started a day after administration of tetrachloromethane. Physiological solution and liposomal drug were injected into the thigh muscle of the pelvic limb three times once each two days.

Throughout the study, the animals were examined clinically. After the treatment, we decapitated the rats and sampled blood and liver tissues for the laboratory and histological analyses. The animals were decapitated under thiopental narcosis one day after the three-time injection of the drug we developed. Blood was collected using cardiotomy of the upper part of the heart. Then, we sampled the liver tissues for histological examinations.

From the blood samples, we made serum and conducted its analysis on a BS-120 automatic biochemical analyzer (Shenzhen Mindray BioMedical Electronics Co., Ltd., China) with PZ Cormay S.A. (Poland) reagents. In blood serum of the rats, we measured contents of albumin, total cholesterol, total bilirubin, and bile acids, and also activity of enzymes – alanine aminotransferase (ALT, KF 2.6.1.2), aspartate aminotransferase (AST, KF 2.6.1.1), gamma-glutamyl transpeptidase (GGT, KF 2.3.2.2), and glutamate dehydrogenase (GLUD, KF 1.4.1.2).

For the histological studies, 0.3–0.5 cm samples of the liver were fixed in 10% formalin solution, dehydrated in alcohols of ascending concentrations, embedded in paraffin. Then, we prepared 5–7 µm-thick sections on a sledge microtome. The prepared sections were attached to the microscopic slides, stained with Ehrlich’s hematoxylin and eosin, and studied histologically using a light microscope, at 200 times magnification.

Statistical analysis of the blood parameters was carried out using the Statistica 7 (StatSoft Inc., USA) software. The graphs were developed in Statistica 7 using the generally accepted algorithms. The paper presents mean arithmetic values and standard error x ± SD (mean ± standard deviation), given in the figures. To compare the difference between the mean parameters between the control and experimental groups, we used the Tukey test, where the differences were considered statistically significant at *P* < 0.05 for all the data.

### Results

The histological study of the liver samples of the rats that had received 5 mL/kg dose of sunflower oil revealed that the organ maintained its structure. Hepatocytes were of regular shape, with homogeneous cytoplasm and centrally located nuclei (Fig. 1a). At the same time, the liver of the animals that had received *CCl*$_4$ intragastrically in 5 mL/kg dose was in the condition of fatty infiltration, with neosis and nuciation of nuclei seen in some places (Fig. 1b). This can be characterized as development of toxic fatty infiltration of the liver (fatty liver disease).

Disorders in the structures of hepatocytes of the rats that had received *CCl*$_4$ were evidenced by high activities of the liver-indicative enzymes. Therefore, in blood serum of the toxic-fatty-steatosis rats, compared with those that received oil, there were significantly (*P* < 0.001) higher activities of AST (26.8 ± 2.5 against 11.6 ± 1.3 U/L), ALT (21.7 ± 3.6 against 8.5 ± 1.4 U/L), GLUD (89.9 ± 3.8 against 11.3 ± 0.7 U/L), and GGT (83.7 ± 7.3 against 7.5 ± 1.4 U/L).

At the same time, in blood serum of the rats that had intragastrically received sunflower oil, relative amount of albumin accounted for 33.5 ± 0.7%. In those that received *CCl*$_4$, it decreased to 29.3 ± 0.2% (*P* < 0.001). Development of pathology in the rats that had been given tetrachloromethane caused impairments in the bile formation and excretion. In blood of the sick animals, concentration of bile acids in blood serum increased to 55.7 ± 7.7 µmol/L, which was five times higher (*P* < 0.001) than in the clinically healthy animals (9.2 ± 0.2 µmol/L). In blood serum of the sick
animals, cholesterol decreased to 0.88 ± 0.14 µmol/L, compared with 2.07 ± 0.09 in the healthy animals (P < 0.001). Also, concentration of total bilirubin increased to 21.6 ± 1.6 µmol/L (P < 0.001) against 9.1 ± 0.8.

Therefore, intragastric administration of tetrachloromethane to the laboratory animals caused development of toxic fatty steatosis, characterized by fatty infiltration of hepatocytes and focal necrosis, impairment in the main liver functions, in particular protein-synthesizing, bile-forming, and bile-excreting functions, and also increased activity of cytolitic (AST, ALT, GLUD) and cholestatic (GGT) enzymes.

The next stage of the studies was analysis of effectiveness of the drug we developed based on extract from S. marianum seeds, complemented by tocopherol acetate, squalene, lecithin, and Tween 80, in the treatment of the rats with experimentally reproduced toxic fatty liver disease. The conducted clinical studies of the animals revealed that three-time injection of the drug led to improvement of their general condition. According to the clinical signs, the best effect was seen in the animals that had received the drug intramuscularly in the doses of 0.25, 0.50, and 1.50 mL/kg of body weight. The rats were more active, had good appearance, and consumed fodder and water better. In somewhat worse clinical condition were the animals that had received the drug in the dose of 0.05 mL/kg of body weight. The rats that had been given the physiological solution were fatigued, poorly consumed fodder and water, the skin coating remained unkempt and pale, and some had diarrhea. Intramuscular injections of the developed drug significantly improved the functional condition of the liver of the sick rats. Therefore, after three-times intramuscular injection of the drug to the sick animals in the doses of 0.05 and 0.25 mL/kg of body weight, we saw increase in the relative content of albumins to 26.3 ± 0.6% (P < 0.05) and 27.8 ± 0.8% (P < 0.05), compared with 22.5 ± 0.6% in those that received physiological solution (Fig. 2a). Increase in the drug doses to 0.5 and 1.5 mL/kg led to improvement of hepatoprotective properties and highly significant (P < 0.001) increase in albumins in blood serum of the animals to 29.9 ± 0.8% and 30.3 ± 1.0%, respectively.

**Fig. 1.** Histology of the liver of the rats after administration of sunflower oil (a), tetrachloromethane (b): hematoxylin and eosin, x 200

Administration of the minimal dose of the drug decreased the content of bile acids in blood by 37.0% (P < 0.001). The next doses normalized the parameters, which were much lower (2.0- and 3.2-fold) (P < 0.001) than in rats of the control group (Fig. 2b).

In the blood serum of the rats with toxic fatty infiltration of the liver which did not receive the drug, the content of total cholesterol was extremely low (Fig. 2c). In blood of the animals that had been receiving the lowest dose of the drug based on extracts from S. marianum seeds, we observed increase in cholesterol content. Higher injection doses promoted significant increase in its content (P < 0.05).

Intramuscular injections of the drug in 0.25, 0.50, and 1.50 mL/kg doses led to decrease in total bilirubin (P < 0.001) in the blood serum, compared with the control animals (Fig. 2d). After administration of the two highest doses, the concentration of total bilirubin in the blood of the experimental animals was more than twice lower than in those that had not received the drug.

The minimal dose (0.05 mL/kg) of the drug given to the sick rats caused decrease (P < 0.05) in the activity of aminotransferases in the blood serum (Fig. 3a, 3b). Increase in administered dose of the drug up to 0.25, 0.50, and 1.50 mL/kg decreased ALT activity in the blood serum by 30–61% and AST activity by 26–51% (P < 0.001–0.01).

Administration of 0.05 mL of the drug per kg of body weight to the sick rats caused no changes in GLUD activity in the blood serum. At the same time, higher doses led to significant decrease in GLUD activity in the blood serum (P < 0.001), compared with the parameters of the sick animals that had received no treatment (Fig. 3c).

Activity of GGT in blood of the animals that received only 0.05 mL/kg of body weight decreased by 14.2%, compared with the control (P < 0.01). Intramuscular injections of higher doses of the drug led to significant (P < 0.001–0.01) decrease in the activity of enzyme in blood serum of the experimental rats (Fig. 3d).

**Fig. 2.** Histostructure of the liver of the rats after administration of sunflower oil (a), tetrachloromethane (b): hematoxylin and eosin, x 200

Results of the histological studies of the animals’ liver after the experiment revealed that the control animals had fatty liver disease (Fig. 4a). At the same time, intramuscular injection of the drug in the dose of 0.05 mL/kg led to partial recovery of the structure of hepatocytes, in particular there was no destruction and no necrotic sites, though in some places we still saw parenchymal fatty infiltration of the liver (Fig. 4b). At the same time, the rats that had been injected the drug in the doses of 0.25, 0.50, and 1.50 mL/kg of body weight had no sites of necrosis of hepatocytes and fatty infiltration of parenchyma, while having significant increase in the number of cells with the normal structure, between which there was young connective tissue with a large content of cellular elements – fibroblasts and fibrocytes (Fig. 4c).

**Discussion**

Intragastric administration of CCl₄ to the rats caused histological changes in the liver parenchyma, characterized by fatty infiltration and focal necrosis. This led to impairment of protein-synthesizing, bile-forming, and bile-excreting functions of the liver of the animals, and also development of cytolysis of hepatocytes with increase of activity of indicator enzymes in the blood – GLUD, AST, ALT, and GGT. Such morphological changes in the liver and disorders of the main functions of hepatocytes can be caused by various etiological factors both in laboratory and agricultural animals (Chermushkin et al., 2020; Zelenina et al., 2022; Zhang et al., 2023).

Taking into account how common liver diseases are among animals, we aimed at development of a drug based on the S. marianum seeds for the treatment of the hepatopathology. Because of its therapeutic efficiency and good bioavailability, extracts from the fruits of S. marianum are most often used as various medical oral forms (pills, capsules, syrups, etc.) for recovery of the functional condition of the liver (Mengesha et al., 2021; Molaei et al., 2022). Despite the fact that S. marianum had proved itself as a highly effective medicinal plant for various liver pathologies, development of medicinal drugs requires further research. In particular, low bioavailability of the main agent – silymarin – when administered perorally...
continues to be an obstacle (Wang et al., 2020). Therefore, we developed an injection form of the drug based on extracts from the fruits of *S. marianum*. This allowed for a parenteral administration of silymarin in the animals, and also other biologically active compounds present in extract from the fruits of *S. marianum*, which have positive effects on the antioxidant and membrane-stabilizing functions, prevent hepatotoxic compounds from entering the liver cells, and enhance rates of hepatocyte regeneration (Bencze-Nagy et al., 2023; Shahin et al., 2023). At the same time, simultaneous administration silymarin with other therapeutic agents can increase its hepatoprotective effect (Yu et al., 2018; Martyshuk et al., 2020; Santamarina et al., 2022; Nehmi-Filho et al., 2023). Taking this into account, our idea was to make a drug from the fruits of *S. marianum* for parenteral administration into a body and increase the therapeutic effect of silymarin by agents that have positive effects in treatments of liver pathologies. We made a drug in the form of liposomal emulsion, containing the extract from the seeds of *S. marianum*, alpha-tocopherol acetate, lecithin, squalene, and Tween 80. Tocopherol exerted positive effects in treatment and prophylaxis of liver diseases (Federico et al., 2019; Juretić et al., 2021). Its inclusion in the drug provided antioxidant protection and supported integrity and stability of hepatocytes, protecting polysaturated fatty acids included in the membranes of the cell (Ungurianu et al., 2021; Vudmaska et al., 2021; Galli et al., 2022). Good membrane-stabilizing action was also exerted by lecithin (Vivechar & Lapovets, 2018). It mitigated fatty liver disease, improved lipid metabolism, normalized the level of cholesterol and fatty acids in blood, and increased absorption of vitamins A, D, E and K in the intestines (Viñado et al., 2019; Liang et al., 2022). Squalene stimulates metabolism, manifests immune-stabilizing and antioxidant actions, positively influences the lipid metabolism, normalizes content of cholesterol and triglycerides, and regulates the content of bile in the body (Lou-Bonafonte et al., 2018; Lozano-Grande et al., 2018). Tween 80 promotes emulsifying and stabilization of liposomal emulsion of a drug (Wahyuni et al., 2020; Ravichandran et al., 2021). This allowed us to combine active agents with various pharmaceutical properties in one injection, and its preparation in the form of liposomal emulsion gave an opportunity to expand the time of its action towards the organism after intramuscular injection.

We conducted biochemical studies of blood serum of the rats with toxic fatty liver disease, which revealed that three-time intramuscular injection of the drug every two days promoted recovery of protein-synthesizing function of hepatocytes. At the same time, not only did the content of albumins in the blood of the rats increase but this was also within the physiological range. Perhaps, this is related to the fact that the fruits of *S. marianum*, as well as other components of the drug, stabilized the biological membranes of the organelles and outer membrane of the liver cells, protecting it from toxins, providing high functional activity, particularly regarding protein synthesis (Bushchenko et al., 2020; Adetuyi et al., 2021).

**Fig. 2.** Biochemical parameters of blood of rats that had received the liposomal drug: *a* – relative content of albumin (%), *b* – concentration of bile acids (µmol/L), *c* – content of total cholesterol (µmol/L), *d* – content of total bilirubin (µmol/L); groups of animals: *I* – control (untreated), *II*, *III*, *IV*, *V* – experimental (treated with the drug in the doses of 0.05, 0.25, 0.50, and 1.50 mL/kg, respectively; n = 5)
Fig. 3. Activity of the enzymes in blood serum of rats after the treatment: 
a – activity of alanine aminotransferase (U/L), b – activity of aspartate
aminotransferase (U/L), c – activity of glutamate dehydrogenase (U/L), d – activity of gamma-glutamyl transpeptidase (U/L); groups of animals:
I – control (untreated), II, III, IV, V – experimental (treated by the drug in the doses of 0.05, 0.25, 0.50, 1.50 mL/kg, respectively; n = 5

Fig. 4. Structure of the liver of the control (a) and experimental rats after administration of 0.05 mL/kg (b) and 0.25 mL/kg (c) doses of the drug; hematoxylin and eosin; x 200
Intramuscular injection of the developed drug turned out to be effective in normalizing bile formation and excretion in rats with toxic fatty liver disease. This indicated changes in bile-acid parameters in blood serum of the rats. Even the minimal dose of the drug caused decrease in the content of bile acid in blood. The best results were produced by injections of 0.25 and 0.50 and 1.50 mL/kg of body weight of the animals. Decrease in the concentration of bile acids in blood serum of the rats after injecting the drug indicates improvement of their removal into bile and elimination of cholestasis (Vlizlo et al., 2021). At the same time, this promotes better metabolism of fat-soluble vitamins, improvement of emulsifying of lipids, and thus increases their bioavailability for breakdown by lipases in the lumen of the intestines. At the same time, three-time administration of the drug to the animals promoted normalization of cholesterol content in the blood serum. Normalization of cholesterol synthesis in the organisms with liver pathology after administration of the drug from S. marianum was also reported by other studies (Mergesla et al., 2021).

Content of total bilirubin in the blood serum was found to be an informative indicator for control of treatment of rats with fatty infiltration of the liver. Three-time administration of the drug decreased the total bilirubin in the blood, and in most animals the parameters corresponded to their levels in the healthy animals. Normalization of content of total bilirubin in blood serum indicated improvement of its absorption by liver cells, conjugation, and removal into bile (Wagner et al., 2018). Therefore, the drug we developed based on extracts from seeds of S. marianum promoted normalization of bile acids, total cholesterol, and total bilirubin in the blood, indicating recovery of bile-forming and bile-excreting functions of the liver. Effectiveness of the drugs prepared from S. marianum seeds was reported by other scientists (Heidarian & Nouri, 2021). In particular, the studies indicated that silymarin promoted transport of bile through the ductal membrane, increased rates of its flow during hepatocellular cholestasis, in vivo stimulated rates of synthesis of bile salts in the rats (Wadhwa et al., 2022). Also, silymarin exerted properties of recovery during fatty liver disease (Zhang et al., 2021). Because the development of fatty infiltration of hepatocytes in the laboratory animals was manifested in cytology with increase in the activity of liver-indicator enzymes, the changes in the activities of AST, ALT, GLUD, and GGT in the blood serum of the sick animals were clearly indicative of the effectiveness of treatment using each dose of the drug. The positive role of association of silymarin and vitamin E in normalization of activities of liver-indicator enzymes (ALT, AST, ALP, and GGT) in blood was also found in other studies (Carcio et al., 2020; Iueng et al., 2022). Furthermore, other than the structure of hepatocytes, the drug also produced positive effect on their mytochondria, because GLUD actively decreased, especially after administration of higher doses. Activity of glutamate dehydrogenase, as a hepatospecific enzyme, changed similarly to AST and ALT, and decrease in the activity indicated stabilization of hepatocytes at the organ level (Armanini et al., 2021; Stoev et al., 2021).

Three-time intramuscular administration of the drug based on the extracts from S. marianum seeds together with tocophorol acetate, lecithin, squalene, and Tween 80 promoted recovery of the liver cells that form intrahepatic bile ducts. This was indicated by changes in GGT activity in blood serum of the rats. It has to be noted that GGT activity, content of bile acids, and bilirubin in blood underwent changes similarly, confirming the effective action of the drug on the bile formation and bile excretion, and also elimination of cholestasis.

Our histological studies of the liver of the rats after the treatment confirmed the positive effect of the drug on the organ structure. This was also found in other studies on models of laboratory animals with the fatty liver pathology (Nehmi et al., 2021). Silymarin administered to the body prevented damage to the liver cells, leading to decrease in the activity of liver-indicator enzymes and improvement of the parenchyma structure (Heidarian & Nouri, 2021). Perhaps, this can be explained by the fact that active agents of S. marianum seeds stabilize the plasmatic membrane of hepatocytes during modeling of liver pathology in laboratory rats using CCl₄ (Javed et al., 2018).

Conclusion

Treatment of the rats with fatty liver disease using three-time intramuscular injection of the developed liposomal drug made from extracts from the seeds of S. marianum, and also tocophorol acetate, lecithin, squalene, and Tween 80, effectively improved the clinical condition, structure, and functions of the liver cells. In blood serum of the rats, there occurred decreases in the activities of AST, ALT, GGT, GLUD, concentrations of total bilirubin, and bile acids, and increases in albumin and cholesterol. The drug exerted positive treatment effect in the doses of 0.25, 0.50 and 1.50 mL/kg of body weight of the animals.

The authors declare no conflicting interests.

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