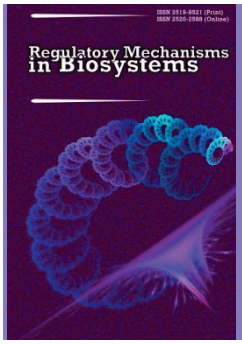

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Treatment of cows with liver pathology using a liposomal drug based on extract from the fruits of *Silybum marianum*

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After labor, dairy cows are often diagnosed with fatty liver disease. The objective of our study was to identify the efficacy of a liposomal drug based on extract from seeds of *Silybum marianum* (L.) Gaertn., including tocopheryl acetate, lecithine, squalene, and Twin-80, which was intramuscularly administered to dairy cows to recover the functional state and structure of the liver from the disorder. The experiment involved clinically healthy cows and cows suffering disorders in the main functions and the structure of the liver. The sick cows were treated with intramuscular injections of the drug. Three-time administration of the liposomal drug, with two days interval between each dose, improved the functional condition and the structure of the damaged liver. Biochemical assays of blood of the cows after treatment revealed improvement of the bile-forming and bile-removing functions of the liver, and also removal of cholestasis, as evidenced by decreased concentrations of uric acids, total and conjugated bilirubin, and lower activity of gamma-glutamyl transpeptidase in serum. Intramuscular injections of the drug in the sick animals reduced the activities of the hepatospecific mitochondrial enzyme glutamate dehydrogenase in the blood serum, and also the indicatory enzymes aspartate aminotransferase and alanine aminotransferase, indicating recovery of the structure of hepatocytes and cessation of cytolysis. After treatment, the sick cows were observed to have upward tendencies in albumin and glucose, which may be interpreted as recovery of the protein-synthesizing and carbohydrate functions of the liver. However, three-time intramuscular injection of the *S. marianum*-based liposomal drug did not lead to complete recovery of the functions and the structure of hepatocytes in the cows suffering fatty liver disease, and therefore further research should be carried out, with longer and more complex therapeutic approaches.

Keywords: *Silybum marianum*; hepatoprotectors; silymarin; tocopherol acetate; lecithin; squalene.

Introduction

During the first week of lactation, dairy cows are often observed to suffer fatty liver disease, or fatty infiltration of the liver (Imhasly et al., 2014; Mylostyvyi et al., 2017; Melendez et al., 2018; Mylostyvyi et al., 2023). The disease causes metabolic disorders in the organism and accompanying pathologies, leading to decline in the productivity and early culling of valuable dairy cows (Gruber & Mansfeld, 2019; Mylostyvyi et al., 2021; Zhang et al., 2022). Treatment of cows with liver pathology includes various therapeutic methods and means (Donkin, 2021; Chirivi et al., 2023). Valuable tools in treating liver diseases are hepatoprotectors, mostly plant-based complex drugs that prevent harmful impacts of various pathogenic factors on the liver cells and promote recovery of metabolism and the functions and the structure of hepatocytes (Gutyj et al., 2017; Slivinska et al., 2019; Li et al., 2021). An effective hepatopotector is *Silybum marianum*, whose beneficial effects are mainly attributed to the biologically active compound silymarin (Bencze-Nagy et al., 2023; Guerrini & Tedesco, 2023). Furthermore, the fruits of *S. marianum* contain fatty (up to 32%) and essential (0.08%) oils, biogenic amines (tyramine, histamine), flavanoids (2.8–3.8% – silibinin, silidianin, taxifolin, and others), cuprum and selenium, and hydro- (group B) and fat-soluble (A, D, E) vitamins (Bashchenko et al., 2020; Guerrini & Tedesco, 2022). Drugs made of *S. marianum* are successfully used to treat patients with liver pathologies, both humans and animals (Wadhwa et al., 2022).

The hepatoprotectory properties of sylimarin and other biologically active compounds of *S. marianum* manifest in positive effects on the structure of hepatocytes and the course of physiological-biochemical processes in them (Zhu et al., 2018; Khazaei et al., 2022). In particular, animal models with fatty liver disease were observed to have improvements in the synthesis of proteins, normalization of the activity of enzymes, and recovery of hepatocytes and their mitochondria (Hackett et al., 2013; Gutyj et al., 2022; Lieshchova & Brygadyrenko, 2023). At the same time, researchers observed enhanced transport of nutrients and biologically active compounds through cellular membranes, in particular, export of bile salts (Adetuyi et al., 2021). Other than hepatoprotecive action, *S. marianum* exerted antioxidant, antimicrobial, immunomodulating, anti-inflammatory, anti-tumor, cardiovascular-protective, neuroprotective, anti-diabetes, and other medical properties (Denev et al., 2020; Islam et al., 2021).

Therefore, extracts from the seeds of *S. marianum* are natural, ecofriendly, multifunctional, and multi-purpose drugs. From the economic perspective, their use is justified by cost-effectiveness and therapeutic benefits, as well as the absence of side effects on the body (Gillessen & Schmidt, 2020). To animals, preparations from seeds of *S. marianum* are mostly given perorally in the form of fodder or food supplements (Khaleghipour et al., 2019; Sobolev et al., 2019; Tedesco & Guerrini, 2022). However, they have low bioavailability for most animals when administered *per os*. This is due to the fact that the main active agent sylimarin contains flavonolignans that are poorly absorbed in the organism when given orally, because they are quickly conjugated in the cells of the intestine and liver and discharged with bile (Tvrdý et al., 2021). In bovine cattle, those processes are especially active in the rumen (Křížová et al., 2011). At the same time, *S. marianum* can alter the enzymatic and micro-

biological processes in the rumen (Liu et al., 2023). Moreover, extracts from the fruits of *S. marianum* are poorly soluble in water, which also hinders their absorption (Abenavoli et al., 2018).

As shown by our previous studies, parenteral administration of extracts from the fruits of *S. marianum* was effective in treating rats with experimentally recreated fatty liver disease (Vlizlo et al., 2023). Therefore, the objective of our study was identifying the efficacy of intramuscular injection of dairy cows with a drug based on extract from seeds of *S. marianum*, also containing tocopherol-acetate, lecithin, squalene, and Tween-80, focusing on recovery of the functional condition and structure of the diseased liver.

Materials and methods

Maintenance, feeding, monitoring, and all the procedures with the animals were carried out 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 conducted adhering to the principles of humanity established in the Directive of the European community.

The studies were conducted on the Ukrainian Black-Spotted dairy cows, aged 4–5 years, 2–3 weeks after labor, with the productivity in the previous lactation equaling 7,100–8,400 L of milk. In order to study the effectiveness of the drug based on the seeds of *S. marianum*, four groups of cows were formed. The first group contained 15 clinically healthy, and the second comprised 30 animals that were ill prior to treatment. The latter was divided into the third and fourth groups, 15 animals in each to carry out the treatment. The third and fourth groups of ill cows were parenterally given our liposomal drug based on seeds of *S. marianum*. The drug was in the form of liposomal emulsion. Hundred milliliters contained 80 mL of aqueous and 15 mL of oil extracts from the *S. marianum* fruits (seeds). The *S. marianum* extracts were supplemented with 1.5 mL of alpha-tocopherol acetate, 0.5 mL of squalene, 3 mL of lecithin, and also 0.4 mL of Tween-80, which – as a surfactant – promoted creation of liposomal emulsion. The mentioned components were mixed and dispersed on an UZDN-1 ultrasound device at the frequency of 22 kHz for 2–3 min until formation of a homogenous emulsion. The drug was administered to the cows intramuscularly into the region of the thigh. The third group received the dose of 25 mL and the fourth received 30 mL per animal, three times, once in 2 days.

The liver pathology in the cows was identified based on the laboratory blood assays. Blood for the studies was withdrawn from the jugular vein before morning feeding. In the first and second groups, blood was collected prior to treatment, and in the third and fourth – the day after the last injection of the drug. From animal blood, we prepared serum and performed studies on an automatic biochemical analyzer BS-120 (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) with the PZ Cormay S.A. (Poland) reagents. In cow blood serum, we measured the contents of total protein, albumin, urea, glucose, total cholesterol, total and conjugated bilirubin, total bile acids, and also the 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 (GLDH, KF 1.4.1.2). To determine the pattern of liver lesion, we carried out veterinary-sanitary examination of the post-partum cows, which were transported to forced or planned slaughter.

The blood parameters were statistically analyzed using a personal computer and the Statistica 7 software (StatSoft Inc., USA). The graphs were developed using Statistica 7 according to the generally adopted algorithms. The paper presents mean and standard deviation ($x \pm SD$). To compare the difference of average 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

Our laboratory assays of blood of the sick cows revealed disorders in the main functions of the liver and heightened activity of enzymes. In particular, compared with the clinically healthy animals, blood serum from the sick cows showed significant increases in uric acids (2.6 times, $P < 0.001$), total bilirubin (2.5 times, $P < 0.001$), and conjugated bilirubin (2.9 times, $P < 0.001$, Fig. 1a). Furthermore, blood of the sick animals had a downward tendency in albumin (Fig. 2b) and low glucose (Fig. 3b). The activity of the liver-indicative enzymes in blood serum of the sick cows was much higher than in the clinically healthy ones (Fig. 4). Therefore, the activity levels of the hepatospecific enzyme GLDH in the blood of sick cows were elevated by 3.8 times ($P < 0.001$), AST by 92% ($P < 0.001$), ALT by 79% ($P < 0.001$), and GGT by 2.0 times ($P < 0.001$).

The veterinary-sanitary examination of the cows after their forced and planned slaughter revealed that liver disorder was characterized by fatty liver disease. Analysis of the assays of blood serum of cows that had received three intramuscular injections of the liposomal drug based on seeds of *S. marianum* revealed declines in the concentration of bile acids, measuring 37% ($P < 0.01$) in the third experimental group, 44% ($P < 0.01$) in the fourth, compared with the parameters of cows prior to treatment (Fig. 1a). However, the levels of bile acids in the blood of animals following drug injections were significantly higher than those in clinically healthy animals, showing an increase of 62% ($P < 0.01$) in those that received 25 mL and 45% in those that received 30 mL.

Usage of the drug led to decrease in the contents of both total and conjugated bilirubin in blood serum of the ill cows (Fig. 1b, 1c). Therefore, administration of 25 mL of the drug led to 31% ($P < 0.01$) decrease in the concentration of total bilirubin in the blood and the dose of 30 mL produced 27% ($P < 0.05$) decrease, compared with the sick cows prior to injection. Compared with the parameters of the clinically healthy cows, the content of total bilirubin in blood serum of the sick animals after treatment was higher by 73% ($P < 0.001$) in the third experimental group and by 83% ($P < 0.001$) in the fourth.

At the same time, content of conjugated bilirubin in the blood serum of the cows (Fig. 1c) that had been injected 25 mL of the drug decreased 2.5 times ($P < 0.001$), and in those that had been injected 30 mL it dropped almost three-fold ($P < 0.001$), compared with the non-treated cows. It has to be noted that the concentration of conjugated fraction of bilirubin in the serum after three injections of the *S. marianum*-based drug did not differ from the parameters of the clinically healthy cows.

In the blood serum of cows of all four groups, the content of total protein during the experiment was almost the same (Fig. 2a). At the same time, the amount of albumin in the serum of the treated cows insignificantly increased compared with the parameters prior to injection, though its level did not correspond to such of the clinically healthy cows.

Treatment of the cows led to no changes in the content of urea in the blood serum (Fig. 3a), but led to insignificant increase in glucose and cholesterol. The *S. marianum*-based drug that had been intramuscularly injected to the sick cows in the doses of 25 and 30 mL per animal produced 41% ($P < 0.01$) and 2.3-fold ($P < 0.001$) decreases, respectively, compared with the parameters prior to treatment (Fig. 4c). After drug injections, the activity of GLDH in blood was even higher in both groups of treated cows (2.5 times in the third, $P < 0.001$, 65% in the fourth, $P < 0.05$) if compared with the clinically healthy cows.

The drug also produced decreases in the AST activity in the blood serum of the sick cows, measuring 26% ($P < 0.05$) in those that received 25 mL dose and 31% ($P < 0.05$) in those that received 30 mL dose. Compared with the parameters of the clinically healthy cows, the activity of the enzyme in blood was higher by 43% ($P < 0.01$) in the third group, and in the fourth the values, although higher by 32%, were statistically unreliable (Fig. 4a). At the same time, the activity of ALT in blood serum of the cows after treatment decreased 25% in the third group and 23% in the fourth, but the values were insignificant compared to the pre-treatment period. At the same time, the values of ALT activity in blood of cows that had received the drug were higher than in the clinically healthy, by 34% ($P < 0.05$) and 39% ($P < 0.05$) in the third and fourth groups, respectively.

In blood serum of the treated cows, the activity of GGT decreased 27% after 25 mL injection ($P < 0.01$) and 33% after 30 mL ($P < 0.01$), compared with the non-treated cows, but the parameters were even higher than the clinically healthy by 53% ($P < 0.01$) and 39% ($P < 0.05$), respectively (Fig. 2d).

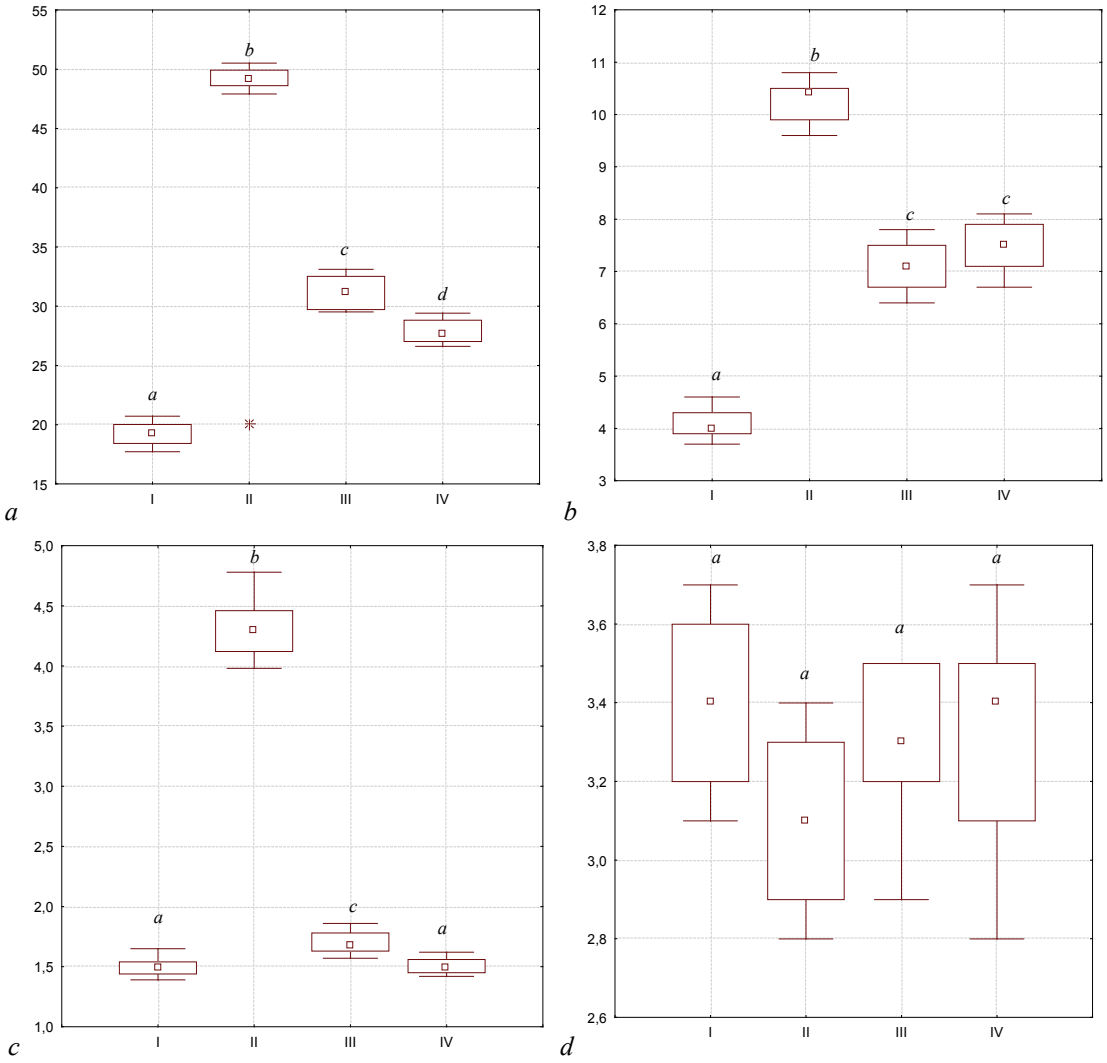

Fig. 1. Biochemical parameters of blood of cows after using the *S. marianum*-based liposomal drug: *a* – content of bile acids (μmol/L); *b* – content of total bilirubin (μmol/L); *c* – content of conjugated bilirubin (μmol/L); *d* – content of cholesterol (mmol/L); groups of animals: I – clinically healthy, II – ill, III, IV – treated with the drug in the doses of 25, 30 mL per animal; different letters within the same system of coordinates indicate samplings that are significantly different one from another according to results of the Tukey Test ($P < 0.05$); small square – median, lower and upper border of rectangle correspond to the first and third quartiles, low and upper border of vertical line correspond to minimal and maximal values

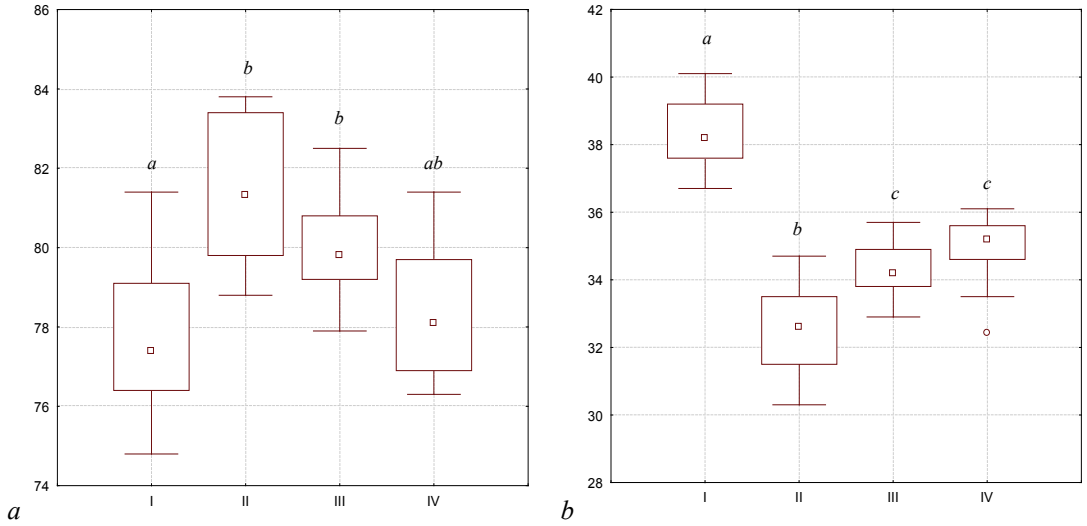

Fig. 2. Contents of total protein and albumin in blood of cows subject to the liposomal drug based on extract from *S. marianum* seeds: *a* – content of total protein (g/L); *b* – content of albumin (g/L); other legends are detailed in Figure 1

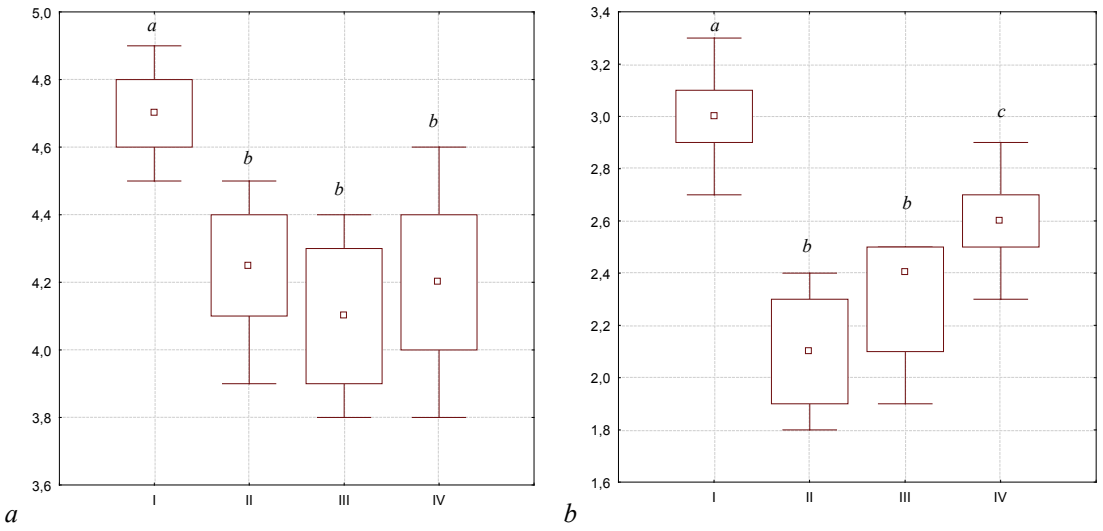

Fig. 3. Content of urea and glucose in blood of cows subject to the liposomal drug based on extract from *S. marianum* seeds: *a* – content of urea (mmol/L); *b* – content of glucose (mmol/L); other legends are detailed in Figure 1

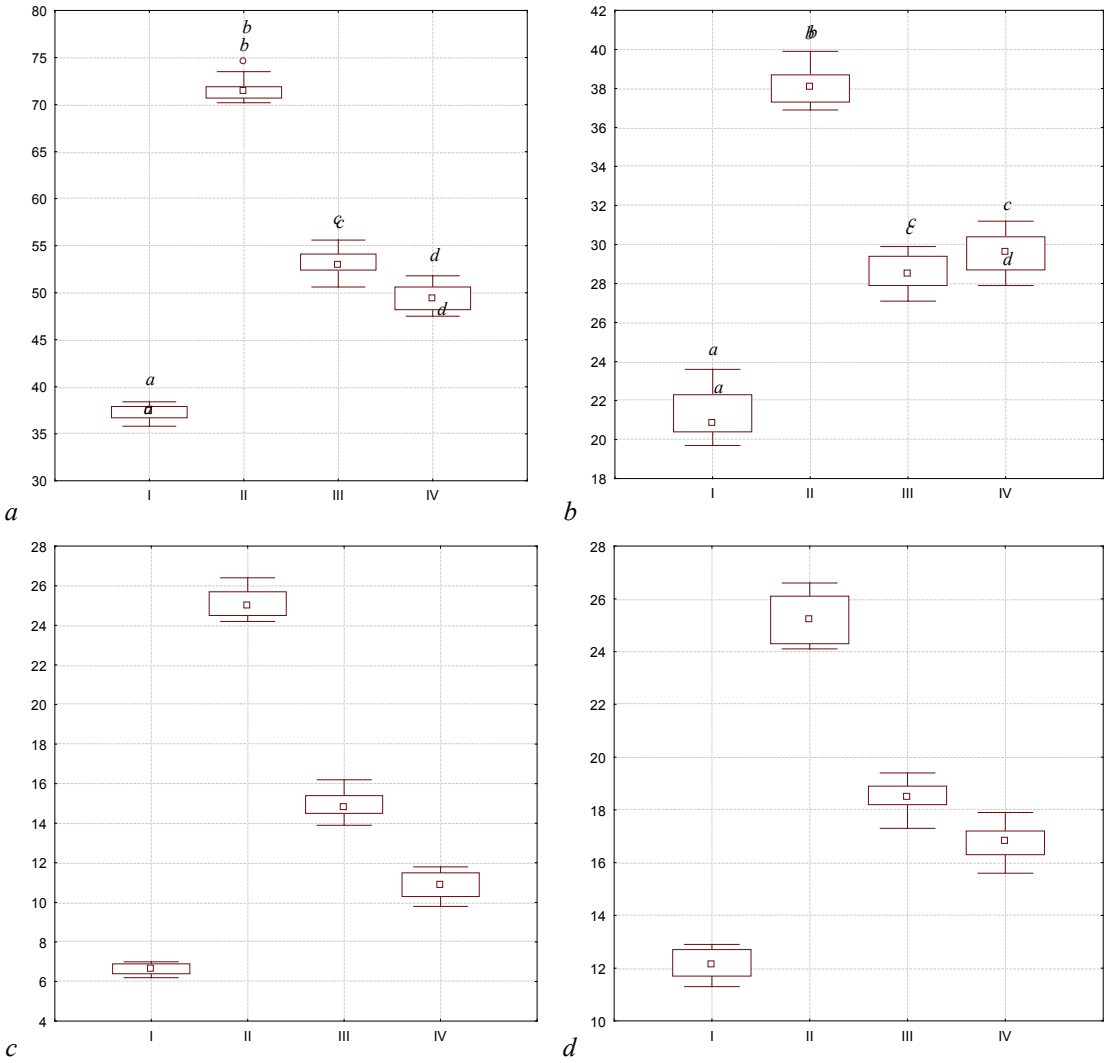

Fig. 4. Activity of enzymes in blood serum of cows: *a* – activity of AST (U/L); *b* – activity of ALT (U/L); *c* – activity of GLDH (U/L); *d* – activity of GGT (U/L); other legends are detailed in Figure 1

Discussion

Fatty liver disease is a common postpartum complication in dairy cows (Slivinska et al., 2018; Turner et al., 2021; Elshafey et al., 2023). As evidenced by our studies, the disease causes disorders of bile formation and bile removal, thus entailing heightened bile acids, total and conjugated bilirubin, and also the activity of GGT in blood serum, indicating damage to liver cells that form intrahepatic bile ducts and development of cholesta-

sis. Fatty infiltration of the liver cells led to elimination of liver-indicative enzymes in blood. Therefore, increased activity of hepatospecific mitochondrial enzyme GLDH in the blood serum suggests disturbed structure and lysis of hepatocyte mitochondria. High activities of AST and ALT were recorded in the blood of all the sick animals. Elevated levels of activities of transaminases in blood serum are observed during development of liver pathology in all species of animals (Chernushkin et al., 2020; Zelenina et al., 2022; Kashliak & Vlizlo, 2023). At the same time, high activities of AST and ALT in blood always occur in patients suffering fatty liver disease (Simonov & Vlizlo, 2015; Bombik et al., 2020).

The treatment of dairy cows suffering from fatty liver disease remains a complex problem with few solutions in veterinary science and practice (Gross, 2023), despite a large arsenal of medicinal drugs. However, when administering drugs to cows, it is important to prevent their influx into the milk, as milk from cows treated with chemicals is prohibited for human consumption (Commission Delegated Regulation (EU) 2019/625 of 4 March 2019 supplementing Regulation (EU) 2017/625 of the European Parliament and of the Council with regard to requirements for the entry into the Union of consignments of certain animals and goods intended for human consumption). Therefore, it is promising to develop nature-derived drugs that would be effective, would quickly mobilize in the organism, and would not accumulate in the mammary gland. For this purpose, we designed a complex liposomal drug of plant origin based on seeds of *S. marianum*.

As evidenced by our previous studies on rats, the drug exerted effective treating properties in cases of experimentally caused toxic liver lesion that leads to fatty liver disease (Vlizlo et al., 2023).

Liposomal emulsion of the drug can considerably reduce the use of active compounds and extend the period of their influence on the organism. Therefore, the drug was administered once in two days. This is especially important to treat animals during lactation, since the stress factor decreases, as well as the productivity of cows after frequent parenteral injections. The way of injection is advantageous due to the fact that administration of drugs from *S. marianum* to ruminants per os causes breakdown of active compounds in the gastrointernal tract and significant decrease in the efficacy of treating properties (Tedesco & Guerrini, 2022). Furthermore, besides silymarin and other active compounds from the seeds of *S. marianum*, the drug also contains alpha-tocopherol acetate, lecithin, squalene, and Tween-80. Tocopherol provides antioxidant protection, promotes recovery of the integrity and stability of hepatocytes (Ungurianu et al., 2021; Vudmaska et al., 2021; Galli et al., 2022). Good membrane-stabilizing action was also exerted by lecithin (Vivchar & Lapovets', 2018). It reduces liver obesity, facilitates metabolism of lipids, normalizes the level of cholesterol and fatty acids in the blood, and enhances absorption of the vitamins A, D, E, and K in the intestine (Viñado et al., 2019; Liang et al., 2022). Squalene stimulates the metabolism of compounds, displays immunostabilizing and antioxidant actions, positively influences the lipid metabolism, normalizes the content of cholesterol and triglycerides, and regulates the content of bile in the organism (Lou-Bonafonte et al., 2018; Lozano-Grande et al., 2018). Tween-80 promotes emulsification and stabilizes the liposomal emulsion of drug (Wahyuni et al., 2020; Ravichandran et al., 2021). Therefore, one injection combines the active compounds with different pharmaceutical properties, while liposomal emulsion of the drug prolonged its action.

Intramuscular three-time administration of the liposomal drug based on extracts from seeds of *S. marianum* to the Spotted cows in the doses of 25 and 30 mL per animal every two days for a total of six days produced positive effects on the patients with liver pathology. After treatment, the experimental cows had improved bile-forming and bile-removing functions of the liver. In particular, normalization occurred in synthesis, conjugation, and removal of bile acids and bilirubin into bile. Injection of the *S. marianum*-based drug led to enhanced removal of bile, which is important in case of cessation of cholestasis (Hackett et al., 2013). After administering the drug three times, the levels of bile acids and total and conjugated bilirubin in blood of cows decreased quite actively, indicating that these parameters can be highly informative control tests of the efficacy of treatment of liver-pathology patients. Recovery of the structure of cells that form intrahepatic bile ducts, improvement of bile removal, and cessation of cholestasis after treatment of cows with the newly formed drug were also indicated by decline in the GGT activity in blood serum. Studies have confirmed the improvement of the protein-synthesizing function of the liver in animals following administration of *S. marianum*-based drugs (Adetuyi et al., 2021; Vlizlo et al., 2023). A tendency towards increase in the content of albumin in blood serum was seen during treatment of the cows, which may be considered an improvement of its synthesis in hepatocytes.

It was reported that addition of supplements from seeds of *S. marianum* to the diet of Hanwoo steers led to improvement of urea formation (Kim et al., 2013). We found no such pattern. However, we had not seen any improvement in urea formation in the sick cows prior to treatment. We should note that synthesis of urea in the liver is quite stable, and its decrease can occur only in case of significant organ lesions (Vlizlo et al., 2021). The liver is one of the main regulators of glucose level in animals. In cows, the need for glucose significantly increases after labor (Vlizlo et al., 2022). Treatment of thesick cows with the drug we developed improved the carbohydrate function of the liver, which was confirmed by the upward tendency in the level of glucose in blood of the animals, though its stabilization requires additional administration of solutions of glucose and glucoplastic drugs (Grummer, 2008; Donkin, 2021).

Treating the cows with liver pathology with the liposomal drug based on *S. marianum* promoted recovery of the structure of hepatocytes. Therefore, the activity of hepatospecific mitochondrial enzyme GLDH in blood serum significantly decreased. We should note that mitochondria play an important role in lipolysis, ketogenesis, and gluconeogenesis, and therefore recovery of their structure and functional state is an indicator of effective action of the drug towards hepatocytes of cows with fatty liver infiltration. It was reported that the administration of drugs made from *S. marianum* to dairy cows with fatty liver infiltration not only led to a recovery of their structure, but also resulted in an increase in their dairy productivity. (Garavaglia et al., 2015; Ulger et al., 2017). Positive effects of the drugs on the liver cells were indicated by the results of studies of other cytologic enzymes in the blood of sick cows. After treatment, the activities of AST and ALT in serum decreased. In our opinion, this correlates with other studies (Guerrini & Tedesco, 2022), and the inhibition of cytolisis and membrane stabilization can be attributed to the positive effects of extracts from *S. marianum* seeds and other constituents of the drug.

Therefore, treatment of cows with fatty infiltration of the liver with the liposomal drug based on *S. marianum* seeds with addition of alpha-tocopherol acetate, squalene, lecithin, and Tween-80 led to improvement of the clinical state, normalization of the functions and structure of the organ, which manifested in decreases in the total and conjugated bilirubin, bile acids, and also the activities of AST, ALT, GLDH, and GGT. Considering that treatment of cows with fatty liver disease using three-time injection of the liposomal *S. marianum*-based drug did not provide complete recovery of the functions and structure of liver cells, further studies should be conducted with longer and more complex therapeutic measures with additional use of other medical products.

Conclusion

The liposomal drug based on *S. marianum*, with addition of alpha-tocopherol acetate, squalene lecithin, and Tween-80 intramuscularly injected in the cows with fatty liver disease three times each two days for six days in the doses of 25 and 30 mL per animal normalized the functions and structure of the liver, which manifested in decrease in the concentrations of total ($P < 0.01$) and conjugated ($P < 0.001$) bilirubin, bile acid ($P < 0.05$), and also the activities of GLDH ($P < 0.01$–0.001), AST ($P < 0.001$), ALT ($P < 0.001$), and GGT ($P<0.01$) in the blood serum.

The authors declared no conflicting interests.

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