



Research article

Blueberry and cranberry extracts mitigate CCL4-induced liver damage, suppressing liver fibrosis, inflammation and oxidative stress



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ABSTRACT

The current study aims to evaluate potential hepatoprotective effect of lingonberry, cranberry and blueberry polyphenols on carbon tetrachloride (CCL-4)-induced acute and subacute liver injury in rats. A total of 55 male Wistar rats, divided into six experimental and control groups. With the exception of the negative control group, all groups received an intraperitoneal injection of CCL-4, twice a week for 14 days. An extract of lingonberry, cranberry, blueberry polyphenols and the positive control, silymarin were administered daily via intragastric route, for 14 consecutive days. The untreated control group showed characteristic of classical oxidative stress-mediated liver damage with vacuolization of the hepatocyte cytoplasm, infiltration by immune cells and proliferation of collagen fibers, decrease in body weight and increase in liver weight; increased levels of AST and ALT in serum, an increased lipid peroxidation in the liver. However, the use of cranberry and blueberry polyphenols significantly suppressed liver damage, exerting an effect comparable to the hepatoprotective effect of the positive control. The extracts prevented and reduced inflammatory liver damage by reducing IL-6, TNF- α and IFN- γ levels. In conclusion, blueberry and cranberry extracts have a protective effect against acute and subacute CCL-4-induced hepatotoxicity in rats.

1. Introduction

Hepatic fibrosis is a highly integrated cellular response to liver injury characterized by the accumulation of extracellular matrix after liver damage, which leads to deformation of the normal structure of the liver and the development of liver cirrhosis or hepatocellular carcinoma [1,2]. Oxidative stress is believed to mediate the onset and progression of liver fibrosis. It has been detected in almost all of the clinical and experimental conditions of liver diseases with different etiology, and the rate of fibrosis progression

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appeared to depend on antioxidant defenses [3]. Excessive accumulation of reactive oxygen species (ROS), destroys macromolecules, causes necrosis and apoptosis of hepatocytes, stimulates the production of pro-fibrogenic mediators, and directly activates hepatic stellate cells, leading to liver damage and induces liver fibrosis [2,4–7].

While advanced liver fibrosis is an irreversible process, the early developmental stages of fibrosis might be reversible [8]. There are no approved pharmacological therapy for the treatment of fibrosis [9]. However, reducing or inhibiting ROS is believed to be one of the potential therapeutic targets for the treatment of early fibrosis. Oxidative stress is the result of an excess level of ROS over the level antioxidant defenses of the organism, which are substances that inhibit ROS or delay oxidation [10]. Therefore, increasing the level of antioxidants may provide an effective and potentially reliable therapy for the treatment of liver fibrosis.

Polyphenols are a significant source of natural antioxidants. They are the most abundant antioxidants in diet and found in high concentrations in fruits including grapes and berries [11]. A number of studies including ours [12], showed the high concentrations of polyphenolic antioxidants extracted from grapes and their potential therapeutic use. While polyphenolic antioxidants from berries such as blueberries are well-investigated, other berries such as cranberry, lingonberry, and blueberry have not been thoroughly described, particularly, their protective effect as antioxidants and therapeutic potential for the treatment of liver fibrosis. Lingonberry, blueberries and cranberries are rich in different phytochemicals and were shown to contain high antioxidant compounds such as anthocyanins, flavonols and chlorogenic acid [13,14]. Several studies described their potential use in the treatment of cardiovascular, cancer and metabolic disorders [15,16]. However, there are no studies that compared their antioxidant activity, polyphenolic contents, or their potential as anti-fibrotic therapy.

Thus, this work aims to determine the polyphenolic concentrates of a different variety of northern berries including cranberry, lingonberry, and blueberry, as well as comparing their anti-fibrotic effects to a standard plant-derived hepatoprotector Silymarin (dry extract of *Silybum marianum*) in a carbon tetrachloride (CCL4)-induced liver damage model.

2. Materials and methods

2.1. Polyphenol extraction

Polyphenols were sourced from wild lingonberry, cranberry and blueberry that were collected in summer-autumn period of 2018 in Surgut district of Khanty-Mansiysk County of the Tyumen region of the Russian Federation. The polyphenolic concentrate obtained from berry skin using water-alcohol feedstock extraction. To extract, a 40% aqueous—alcoholic solution of ethyl alcohol in the ratio 1:5 was used, followed by concentrating the extract on a rotary evaporator to a dry matter content of 25%. The final concentration of phenolic derivatives of berry is of 10 mg/ml. Concentration of polyphenols in samples were determined using a commercially available kit “Polyphenols Folin-Ciocalteu” (ENOLOGY line by BioSyttems. Spain) according to the manufacturing guidelines. In principle, polyphenols in the sample would react with Folin-Ciocalteu reagent in basic media, which leads to an increase of color intensity proportional to polyphenols' concentration in the sample. Briefly, 12 μ l of the sample and 800 μ l of the Folin-Ciocalteu reagent were pipetted into a cuvette. For the negative control (blank), a 12 μ l of distilled water was instead of the test sample and for the standard, Gallic acid solution (2000 mg/L) was used. The reaction mixture was stirred and incubated for 2 min at room temperature, and then a reagent containing sodium carbonate (pH 13.0) was added. The reaction mixture was stirred and incubated for further 8 min at room temperature. After that, the absorbance of a blank sample, standard and test samples were measured at a wavelength of 670 nm. The concentration of polyphenols in the test sample was determined by the formula: $(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{standard}} - A_{\text{blank}}) * 2000$ (mg/L). Silymarin (dry extract of *Silybum marianum*) was obtained from Sigma-Aldrich (S0292, Sigma-Aldrich).

2.2. HPLC analyses

Analysis of the content of phenolic compounds was carried out by high performance liquid chromatography using an Agilent 1290 Infinity chromatograph (Agilent Technologies, Santa Clara, CA), according to our previously published protocol [17].

2.3. Animals

Healthy adult male Wistar rats (outbred albino rats) aged between 10 and 12 weeks and weighing 200 ± 20 g were housed in the animal facility of the National Center for Biotechnology, Astana, Kazakhstan. After one-week adaptation period, the rats ($n = 60$) were randomly divided into 6 groups (10 rats/cage) and housed in a room with controlled temperature and a 12-h light–dark cycle with unlimited access to standard food (SSNIFF V1534-300, HTLab AG, Heideck, Germany) and drinking water ad libitum.

2.4. Treatment protocol

There were six animal groups; the first three experimental groups were used as controls. Group I (negative control-healthy); group II (model of liver pathology, no-treatment control group); and group III (positive control group), where rats were injected with the silymarin drug for comparison. The second set of the three experimental groups each received extracts from different berry. For example, group IV injected with lingonberry concentrate, group V injected with cranberry concentrate, and group VI received blueberry concentrate.

The researched polyphenol concentrates and the reference drug were administered intragastrically through a probe on a daily basis for 14 days. The doses of administrated polyphenol concentrates were 10 mg/kg (volume of injection 0.2–0.3 ml), which was based on

similar studies [18]. The drug of comparison - Silymarin Hexal AG (dry extract of milk thistle) was administered through a gastric probe at a dose of 200 mg/kg, preliminarily suspended in a starch suspension. The administered volume per rat was 0.2–0.3 ml of suspension. Group I (healthy group) and control group II, were intragastrically administered 0.2 ml of saline solution as a placebo on a daily basis.

Group I, received an intraperitoneal injection of saline solution 3 h after the first intragastric administration, and the groups II–VI received an injection of CCl₄ solution to simulate toxic damage to liver tissue. CCl₄ administration was repeated twice a week (Monday - Thursday) for 2 weeks. Liver damage was induced in rats by intraperitoneal injection of CCl₄ with 0.2 ml/100 g of body weight in refined olive oil (1:1) twice a week for 14 days as described by Constandinou et al. (2005) [4].

2.5. Blood collection and analysis of biochemical parameters

After euthanasia of animals by carbon dioxide (CO₂) inhalation using a rodent euthanasia unit (Open Science, AE0904), and then blood was collected from animals in vacutainers with EDTA from the unpaired posterior vena cava (vena cava posterior). The extent of liver injury was determined via measuring the serum level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are commonly used markers of liver injury [19]. The levels of the two markers were determined using commercially available kits AsAT-Vital kit (Vital Diagnostics SPb, Russia), and Alanine aminotransferase Activity Assay Kit (sigma-Aldrich), respectively. Another measured marker of hepatitis injury was total cholesterol (TC) and triglycerides (TG), which was determined using the colorimetric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). The levels of cytokines were analyzed in blood serum, according to manufacturer's instructions, from specific rat ELISA kits for TNF- α , IL-6 and IFN- γ (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Tissue preparation and assessment of lipid peroxidation

Liver samples were isolated, washed, and weighed to determine the liver index, which was calculated as described by Yang et al. (2005) using the following formula: (mass of liver/mass of rat) \times 100% [20]. After dissection, liver tissue samples were washed in 0.9% NaCl solution, and then a sample of approximately 1 g was homogenized on ice into 1: 3 phosphate-buffered saline buffer solution (weight/volume; 1 g tissue with 3 ml PBS, pH 7,4) and centrifuged at 10,000 \times g for 15 min at 4 °C. The supernatants were collected in order to determine the malondialdehyde (MDA) content. The supernatant of liver tissue homogenate was also used to determine the process of lipid peroxidation by the thiobarbituric acid (TBA) method, which evaluates the formation of MDA. The absorption of the TBA-MDA complex was estimated at 530 nm and the MDA level was expressed as μ mol/g [21].

2.7. Histopathological examination

For histological examination, tissue samples were taken from the cross-section of the central part of the right lobe of the rat liver. Hematoxylin and eosin staining was used to determine the general tissue morphology.

Liver tissues were fixed in 10% formalin at 4 °C for 24 h, then washed with tap water and dehydrated by a series of alcohol solutions in ascending concentrations (70%, 90%, 95%, 100%), then were immersed in xylene and poured into paraffin blocks. Tissue sections with a thickness of 5 μ m were made on a "Leica SM 2000R" sledge microtome. The sections were washed three times with tap water and stained with hematoxylin for 40 s. The sections were dewaxed in xylene and then dehydrated in a series of alcohols with descending concentrations (100%, 96%, and 70%), and then stained with eosin. Finally, the sections were treated with xylene and fixed using a mounting medium.

Morphometric analysis was performed by two independent researchers on 10 histological sections. At \times 100 and \times 200 magnifications, the number of vacuolated hepatocytes (per square millimeter) were counted and the fraction of the surface infiltrated by lymphocytes (defined as the ratio of the infiltration area to the total investigated surface of the liver) were determined.

Histological evaluation of liver fibrosis was determined using van Gieson staining by picrofuchsin. Briefly, dewaxed and hydrated sections were immersed in 70% ethanol containing 1% hydrogen chloride, incubated in a resorcinol-fuchsin solution for 60 min, and washed in 100% ethanol and water, followed by staining with van Gieson's solution (saturated picric acid that contains 0.09% sour fuchsin) for 5 min.

Histopathological analysis was performed on the cross-section of the liver, individually, by random sampling for each animal in each group using 10 fields of view. Semi-quantitative counting was performed with the use of a system adapted from METAVIR, differentiating the stages (F-scores) from F0 to F4, where F0: indicates no fibrosis, F1: mild fibrosis (portal fibrosis without septa), F2: moderate fibrosis (few septa), F3: severe fibrosis (numerous septa without cirrhosis), and F4: cirrhosis [22]. The histological examination of the samples was carried out blindly.

2.8. Statistical analysis

The results are presented as "average value \pm standard error of the average value". The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey comparison.

Test. *P*-value less than 0.05 was considered significant.

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the National center for biotechnology (IRB00013497 National Center of Biotechnology IRB #1). The authors confirm that all experiments were performed in accordance with relevant guidelines and regulations. All research work with laboratory animals was performed in accordance with generally accepted ethical standards for the treatment of animals based on standard operating procedures that comply with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes.

3. Results

3.1. Determination of total phenolics and anthocyanins

HPLC chromatograms, composition of anthocyanins and phenolic compounds in berry extracts are attached in appendix 1. Analyses of berry extracts content is available in our previously published article [17].

3.2. Morphology and morphometry

CCl₄-induced hepatotoxicity in rats was assessed using hematoxylin and eosin-stained sections. In the healthy negative control group (Group I), the liver structure corresponded to the histological norm: hepatocytes are located radially with the center in the central vein, there were no signs of dystrophic changes or necrosis of hepatocytes, as well as infiltration by inflammatory cells in the portal tracts (Fig. 1A).

In the control group (model of liver pathology, no-treatment control group II), a part of hepatocytes in the periportal tracts showed edema, dystrophic changes, venous congestion in the central vein and moderate infiltration of portal tracts by immune cells were observed (Fig. 1B). The degree of damage to hepatocytes, determined by the number of hepatocytes with vacuolization, which was 195 ± 54 vacuolated hepatocytes per mm², the area of tissue infiltrated by lymphocytes was 13.5 ± 6.15 (Table 2).

In the positive control group (Group III that received silymarin), the structure of hepatocytes corresponded to the morphological norm, the number of vacuolated hepatocytes per mm² was 8.9 ± 2.2 , and there was a slight periportal infiltration by immune cells (1.1 ± 0.42) (Fig. 1C).

In the lingonberry extract treated group (IV), there was marked vacuolization (124 ± 32), edema and dystrophic changes of hepatocytes in a small area of tissue infiltrated by immune cells (5.6 ± 4.11) (Fig. 1D).

In the fifth group (V) that received cranberry extracts and the sixth group (VI) that received blueberry extracts, the morphological

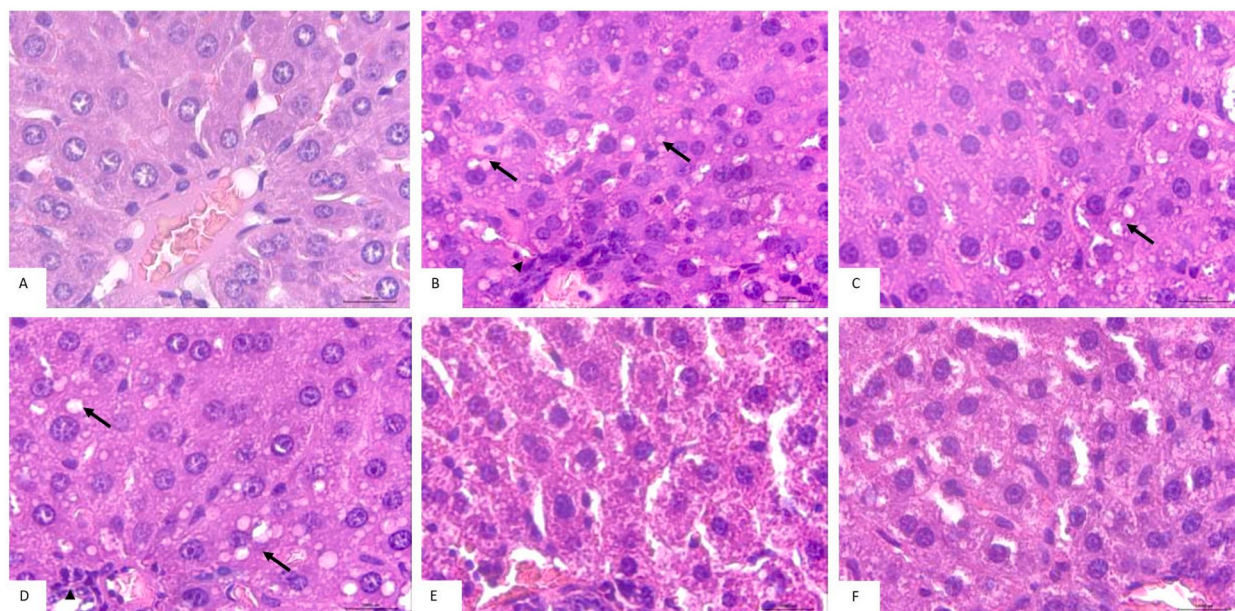


Fig. 1. Evaluation of the effect of berry extracts on the reduction of CCl₄-induced liver damage. X400, H&E. A. Intact group, morphological pattern is saved. B. Control group, pale stained hepatocytes with cytoplasmic vacuolization (arrows) located diffusely. C. Silymarin, vacuolated hepatocytes (arrows) located focally, single immune cells (arrowhead). D. Lingonberry extract, morphological pattern is saved, hepatocyte vacuolization (arrows), individual immune cells (arrowhead). E. Cranberry extract, mild venous stasis, hepatocytes are close to the histological norm in morphological structure. F. Blueberry extract, slight venous stasis, hepatocytes are close to the histological norm in morphological structure, single apoptotic bodies.

Table 1
Concentration levels of the main polyphenol components in the northern berries' extracts.

Sample	Polyphenol content mgGAE/ml
Lingonberry extract	16,3 ± 1,7
Cranberry extract	14,6 ± 0,9
Blueberry extract	29,1 ± 1,4

Note: results are presented as arithmetic mean ± standard error of the mean.

Table 2

The effect of Lingonberry, cranberry and blueberry extracts on lymphocyte infiltration and the density of vacuolated hepatocytes.

Test groups	Vacuolization	Infiltration
Intact rats (norm) (n = 10)	–	–
Control (Fibrosis model) (n = 6)	195 ± 54*	13,5 ± 6,15*
Silymarin (n = 10)	8,9 ± 2,2	1,1 ± 0,42
Lingonberry extract (n = 10)	124 ± 32*	5,6 ± 4,11
Cranberry extract (n = 9)	–	–
Blueberry extract (n = 10)	–	3,2 ± 0,91

Results are presented as arithmetic mean ± standard error of the mean.

Infiltration = ratio of area, infiltrated by lymphocytes, to total examined area; vacuolization = number of vacuolated hepatocytes (density) per 1 mm², «-» absence of feature.

* - p < 0,05 when comparing to intact group; # - p < 0,05 when compared to control (fibrosis model).

pattern of the liver structure was preserved, no histopathological signs of dystrophic changes, vacuolization or edema were observed (Fig. 1E and F, respectively). In the group with the use of blueberry, there was a slight infiltration by immune cells (3.2 ± 0.91) (Fig. 1F). Interestingly there was a significant difference in the severity of fibrotic damage between each of the groups (Fig. 2 and Fig. 3A–F).

In all of the 10 animals in the healthy group (100%), histologically normal structure was determined in all sections of the liver; native collagen fibers were found in the portal tracts and hepatic veins, and thus designated as (F0).

In the second control group, all the animals developed liver fibrosis. One of the animals in this group showed perivascular fibrosis without septa formation (F1). Five animals showed proliferation of collagen fibers in the area of the portal vein with the formation of fibrous septa, both singular and plural, uniting with each other to form false lobules, which is characterized as significant fibrosis (stages F2–F4). Also, another animal showed proliferation of collagen fibers with the formation of singular septa (F2), and in 3 other cases formation of plural septa (F3) were observed. Interestingly, one rat was found to have cirrhosis with the formation of false lobules and bridge-like fibroses that connect the vascular structures (F4).

In group III (silymarin group), 8 of the experimental rats showed small amount of collagen formation, mainly in the portal vein region, with a small distribution in some portal tracts, and the formation of short fibrous sept, which corresponds to the stages F0 and F1. One animal displayed slight fibrosis with single septa (F2), and another animal showed fibrosis with multiple septa without cirrhosis (F3).

However, in the lingonberry extract group, two animals showed no signs of fibrosis, and three rats had perivascular fibrosis without the formation of septa (F1). In the remaining five animals, two animals, showed the formation of small amount of fibrous fibers with the formation of short singular fibrous septa (F1) and the formation of fibrous fibers in moderate amount with the formation of plural septa in the remaining three animals in this group (F2).

In the fifth group (cranberry extract group), a total of eight animals showed absence (5 animals) or mild fibrosis (3 animals) without the formation of septa. The remaining animal in this group, showed perivascular fibrosis without formation of septa.

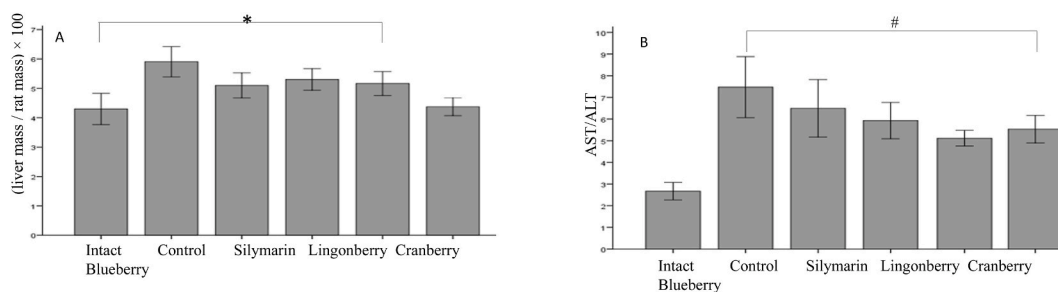


Fig. 2. A. The effect of polyphenols from northern berries on liver index in rats with liver damage, caused by CCl₄ B. AST/ALT ratio. Arithmetic mean ± standard error of the mean * - p < 0,05 when compared to intact group; # - p < 0,05 when compared to control (fibrosis model).

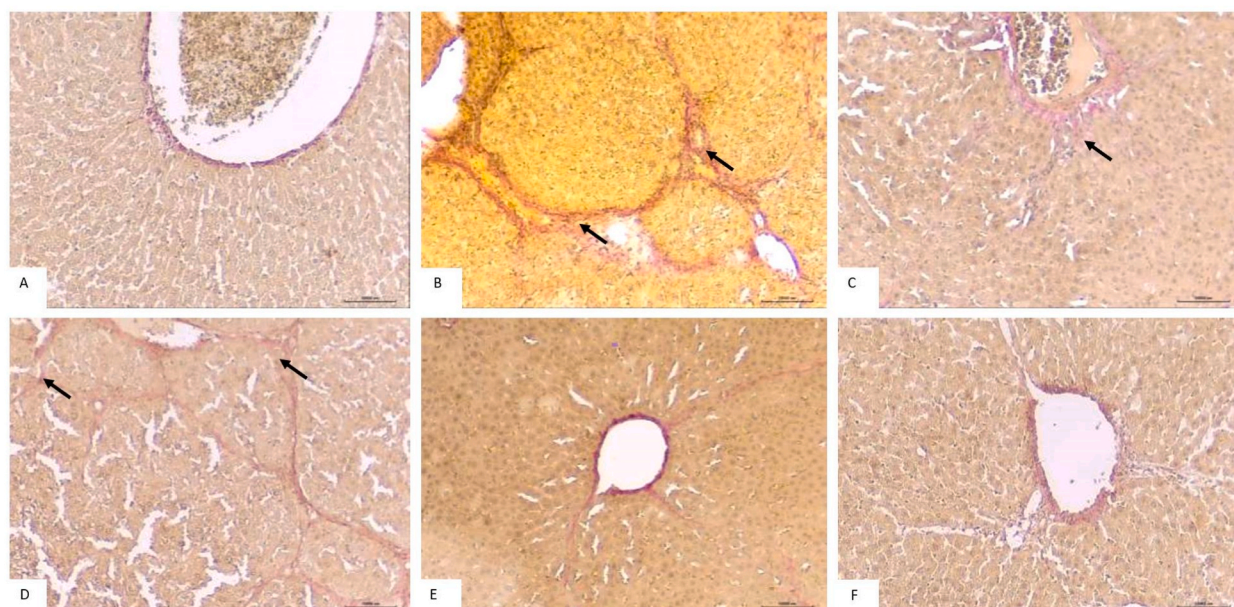


Fig. 3. Micrographs of representative sections of the liver. X100, Staining: Van Gieson. A. Intact Group, histologically normal structure, native collagen fibers in portal ducts and hepatic veins. B. Control group, proliferation of collagen fibers with formation of fibrous cords (arrows) that invade deep into the parenchyma. C. Silymarin, native perivascular proliferation of collagen fibers. D. Lingonberry extract, proliferation of collagen fibers with the formation of fibrous strands (arrows) that invade deep into the parenchyma. E. Cranberry extract, native perivascular proliferation of collagen fibers. F. Blueberry extract, native perivascular proliferation of collagen fibers.

In group VI (blueberry extract group), the stage of liver damage corresponding to F0–F1 was observed in nine animals, five of which showed histological structure corresponded to norm (F0), and in 4 cases displayed native perivascular proliferation of collagen fibers and perivascular fibrosis without the formation of septa (F1). One animal, showed the proliferation of collagen fibers with the formation of singular septa (F2).

3.3. Characterization of body mass, liver mass and biochemical parameters of blood serum

Quantitative characteristics of body mass, liver index and biochemical parameters of blood serum in rats with CCl₄-induced liver damage are presented in Table 3 and Fig. 4.

In group I, the healthy control animals, the average body mass was 232.9 g, and the liver mass was 9.7 g. The average level of ALT and AST in the blood serum was 12.4 IU/L and 5.4 IU/L, respectively and of TG and TC - 5260.2 mg/dL and 114.7 mg/dL, respectively.

In the second control group, the average body mass of rats was 185.5 g, and the liver mass was 11.0 g. Blood serum showed significant increase in ALT and AST levels approximately 12.1 and 5.4 times that of the healthy group, respectively. Also, the level of TG was shown to be 2.7 times that of the health group, and in TC was approximately 4.7 times more.

However, the average body mass of the silymarin treated group was 236.3 g, and the liver mass was 10.9 g. In comparison to control (healthy group), blood serum analysis of animals in this group showed significant increase in the levels of ALT approximately 4.3 times of control; AST about 2 times control; TG approximately 1.9 times of the control and TC about 4.3 times of the control animals.

The average body mass of the lingonberry extract treated rats was 218.4 g, and the liver mass was 11.6 g. The blood serum analysis showed that ALT increased by 8.5 times and AST by 3.1 times compared to the healthy group, as well as TG and TC increased by 2.1

Table 3

The effect of polyphenols from northern berries on body mass, liver mass and biochemical parameters in rats with liver damage, caused by CCl₄.

Test groups	Body mass	Liver mass	AST (U/L)	ALT (U/L)	TG (mg/dL)	TC (mg/dL)
Intact rats (norm) (n = 10)	232,9 ± 8,42	9,7 ± 1,22	12,4 ± 1,17	5,4 ± 0,87	5260,2 ± 94,20	114,7 ± 9,44
Control (fibrosis model) (n = 6)	185,5 ± 6,15*	11,0 ± 0,92	149,8 ± 12,60*	28,9 ± 2,17 *	14,386,5 ± 104,37*	535,3 ± 22,18 *
Silymarin (n = 10)	236,3 ± 5,48	10,9 ± 1,10	53,2 ± 6,73* #	10,6 ± 1,76 **	9763,4 ± 160,45 #	490,7 ± 20,44
Lingonberry extract (n = 10)	218,4 ± 7,11	11,6 ± 0,97	104,9 ± 11,62*	16,9 ± 1,65*#	11,053,2 ± 144,62 #	480,4 ± 29,80#
Cranberry extract (n = 9)	198,6 ± 9,14	10,5 ± 0,73	97,4 ± 5,73*	17,3 ± 2,04 **	10,534,7 ± 122,60 #	398,5 ± 29,46 #
Blueberry extract (n = 10)	233,2 ± 9,08	10,3 ± 1,24	58,3 ± 4,82 **	11,2 ± 2,05 **	8060,0 ± 207,16 #	240,8 ± 16,70#

Note: AST – aspartate aminotransferase; ALT – alanine aminotransferase; TG - triglyceride; TC – total cholesterol.

* - p < 0,05 when compared to intact group; # - p < 0,05 when compared to control (fibrosis model).

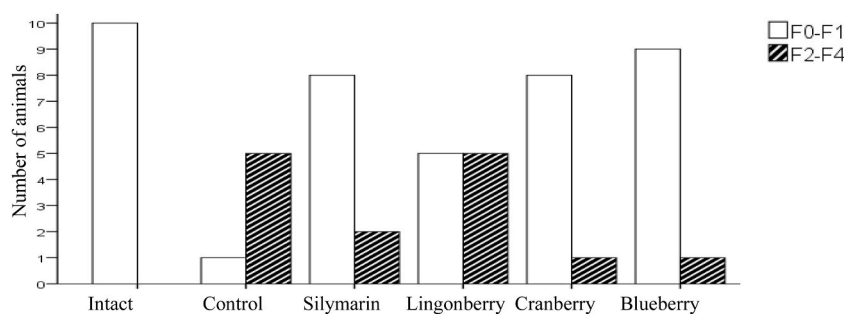


Fig. 4. Morphological assessment of CCl4-induced liver fibrosis in rats.

times and 4.2 times of the control group, respectively.

The average body mass in group V (cranberry extract treated animals) was 198.6 g, and the liver mass was 10.5 g. An increase in the ALT level to approximately 7.9 times of the control was observed. Also, the AST level was 3.2 times compared to the control group, TG level was 2 times that of the control and the TC level was measured as 3.5 times more than control group.

The blueberry extract treated animals had an average body mass of 233.2 g, and average liver mass of 10.3 g. The blood serum showed an increase in ALT levels of approximately 4.7 times control and AST by 2.1 times the control. Also, TG and TC levels were increased by 1.5 times and 2.1 times of the control, respectively.

3.4. Characterization of pro-inflammatory cytokines and oxidative stress

The average level of IL-6, TNF- α and IFN- γ cytokines in the healthy group were 23.0 pg/ml, 318.8 pg/ml, and 38.14 pg/ml, respectively and the mean MDA level in the blood serum of was 0.62 μ mol/L (Table 4). In the second control group, the average level of IL-6, TNF- α and IFN- γ cytokines were 2.6, 2.3, and 2.1 times higher than those of the healthy group respectively. The mean MDA level in the blood serum was estimated to be 4.2 times higher than the control group.

In group III (silymarin treated group), the average level of cytokines was 1.6 times higher than the healthy group for IL-6, 1.5 times higher for TNF- α , and 1.6 times higher for IFN- γ , and the mean level of MDA in the blood serum was 3.5 times higher.

The average level of IL-6, TNF- α and IFN- γ cytokines in the lingonberry treated group were 2.2, 1.3, and 1.8 times higher than those of the healthy group respectively, with the mean MDA level was 2.9 times higher than the healthy control group. In group V (cranberry extract group), the average level of IL-6 was 2 times higher, TNF- α was 1.6 times higher and IFN- γ was 1.7 times higher than those of the healthy group, and the mean MDA level was 2.2 times of the healthy control. The blueberry extract group had an average level of IL-6 approximately 1.4 times of healthy control, TNF- α about 1.2 times healthy control, and IFN- γ cytokines were approximately 1.3 times higher than those of the healthy group. The mean MDA level in the blood in this group exceeded the mean level of the healthy group by approximately 2.5 times.

4. Discussion

The current manuscript is an investigation of the antifibrotic potential of lingonberry, cranberry and blueberry extracts by estimating several pharmacodynamics, biochemical and pathomorphological parameters in rats with CCl4 induced liver fibrosis, which is a widely used and accepted model for the study of hepatic fibrosis and screening of hepatoprotective [23]). We have previously shown the cytoprotective effects of berry polyphenols solutions on myocardium [24]. Interestingly, the results from the current research demonstrate similar benefits from cranberry and blueberry extracts on liver damage. Blueberry and Cranberry extracts reduced CCl4-induced liver damage by decreasing fibrosis, reducing inflammation and oxidative stress.

The current findings confirm the anti-fibrotic effect of blueberry and cranberry extracts and that the differences in the histological severity of fibrosis between the non-treated control group and the groups that were treated with blueberries and cranberries extracts were statistically significant ($p < 0.05$). The histological analysis of these two groups were comparable with the silymarin treated group, further confirming the anti-fibrotic potential of blueberries and cranberries extracts against the CCL4-induced liver damage.

Table 4

The levels of IL-6, TNF- α and IFN- γ cytokines in rat blood serum and MDA in rat liver tissue.

Test groups	IL-6 (pg/mL)	TNF- α (pg/mL)	IFN- γ (pg/mL)	MDA (μ mol/L)
Intact rats (norm) (n = 10)	23,0 \pm 1,17	318,8 \pm 22,65	38,14 \pm 2,26	0,62 \pm 0,07
Control (fibrosis model) (n = 6)	59,5 \pm 4,56*	733,5 \pm 40,27*	81,3 \pm 6,72*	2,62 \pm 0,08*
Silymarin (n = 10)	36,9 \pm 3,22#	488,4 \pm 20,41 #	59,36 \pm 4,02 #	2,15 \pm 0,11#
Lingonberry extract (n = 10)	49,6 \pm 4,22	404,2 \pm 47,83 #	70,5 \pm 4,88	1,80 \pm 0,09#
Cranberry extract (n = 9)	46,3 \pm 2,81 #	501,7 \pm 33,61 #	65,4 \pm 4,37	1,38 \pm 0,05#
Blueberry extract (n = 10)	33,1 \pm 2,49 #	391,7 \pm 25,14 #	51,1 \pm 4,38 #	1.55 \pm 0,04#

These findings are in line with previously published work that investigated anti-fibrotic effects of berries, including Sun et al. which studied the antioxidant effects of anthocynins, on CCL4-induced liver fibrosis on mice [25].

The morphological analysis of the CCL4-induced fibrosis model of the non-treated control group, showed a diffused and widespread pattern of damage with pronounced collagen proliferation, and formation of fibrous septa that disrupts the structure of the liver tissue that formed false lobules (F3–F4). However, in the blueberries and cranberries treated groups, fibrosis was absent (F0) or had a focal mild nature (F1), which indicates the ability of these extracts to suppress the development of liver fibrosis in liver damage.

The METAVIR scoring system, which was used to assess the stage of fibrosis, showed histological improvements in liver fibrosis in both of the cranberry and blueberry extract treated groups. Additionally, Van Gieson histological staining, showed that the administration of these extracts led to a significant improvement in liver fibrosis compared with the control group ($p < 0.05$). The improvement was likely due to the anti-fibrotic activity of berry extracts that might have occurred due to the reduction in inflammation and oxidative stress. The antioxidant and anti-inflammatory activity of berries reported both in animal and in vitro studies [26, 27]. However, the regenerative capabilities and resolution of liver fibrosis were significantly more effective with the use of blueberry and cranberry extracts than with silymarin and lingonberry.

Furthermore, serum from CCl4 treated rats, showed a significant increase in the AST and ALT levels, but treatment with berry extracts significantly reversed this effect, confirming the hepatoprotective effect of these extracts. While the non-treated CCL4 group, showed restriction of cytolytic processes (characterized by decrease in ALT and AST levels), all of the berry extracts, particularly blueberry treated animals, were able to significantly reverse this effect.

Importantly, the results confirmed that cranberries and blueberries are able prevent and reduce inflammatory liver damage in rats by lowering IL-6, TNF- α and IFN- γ levels. Similarly, a study by Kowalska et al. who investigated the anti-inflammatory effect of lingonberry in mouse adipose, reported potential anti-inflammatory effect lingonberry through the reduction of a number of proinflammatory cytokines including IL-6, TNF- α , and IL-1 β [28]. However, as far as we know, this is the first study to report and confirm the anti-inflammatory activity of cranberries and blueberries.

While, the results also confirmed the antioxidant activity of the tested berries, the exact mechanisms underlying the protective effects remain undetermined. A possible mechanism that may have contributed to the observed anti-fibrotic activity of the northern berry extracts on the CCl4 model of liver fibrosis is the antioxidant ability of the extracts. However, another possible theory about the antifibrotic mechanism is through the interaction of polyphenols from berries with intestinal microbiota. Considering the role of the gut–liver axis in the development of liver fibrosis and the importance of gut microbiota homeostasis [29–31], we speculate that lingonberry; cranberry and blueberry extracts may achieve their anti-fibrotic activity by improving and maintaining the integrity of intestinal epithelial, and gut microbiota. Previously, we showed that plant polyphenols can significantly influence and change intestinal microbiota [32]. Nevertheless, further research is needed to decipher the exact protective mechanism of blueberry and cranberry extracts against CCl4-induced liver damage.

Furthermore, several reports indicated that the underlying defense mechanisms of blueberry extract may include the ability to scavenge free radicals, anti-apoptotic and anticarcinogenic effects [18,33–35]. These reports support our finding that blueberry extract is an effective antioxidant and free radical scavenger that can prevent ROS formation and have a protective effect on hepatocytes.

In this study, the antioxidant activity of berry extracts was identified by using oxidative markers such as MDA, which is a lipid peroxidation product that is released during oxidative stress. The results showed that increased lipid peroxidation, accompanied by a deterioration in antioxidant status was apparent in the control group. However, the use of both silymarin or berry extracts significantly reduced the oxidative stress caused by CCl4 ($p < 0.05$), this effect was most pronounced with the use of cranberry extract. The use of lingonberry, blueberry, and to the greatest extent cranberry extracts, decreased CCl4-induced liver damage. This effect is most likely due to their potent antioxidant property and their ability reverse the oxidative stress-induced liver damage [27,36,37].

The administration of CCl4 led to the formation of a pattern of liver tissue damage, which was manifested in a decrease in body mass, increase in liver mass, and an increase in liver index [23,25,38], which are the typical characteristics of CCl4-induced liver damage. These effects were also accompanied by increase in the serum concentrations of AST, ALT, TG and TC, as well as the levels of IL-6, TNF- α and IFN- γ proinflammatory cytokines [39]. The level of lipid peroxidation MDA products was also significantly increased. Morphologically, liver damaged was expressed through the emergence of signs of fibrosis in the control group determined by Van Gieson staining including the presence of collagen fibers around the central vein and the pronounced proliferation of the portal area with the formation of fibrous septa that disrupt the structure of the liver tissue and formed false lobules.

This study confirms the protective effects of blueberry and cranberry extracts against CCl4-induced fibrotic liver damage in rats. The use of these extracts was effective in reducing fibrosis, inflammation and oxidative stress that accompany CCl4 treatment. The findings suggest that administration and possibly combination of these extracts may provide a potential adjuvant therapeutic approach capable of mitigating CCl4-induced liver damage. Further research is needed to investigate the safety and efficacy of the northern berry extracts as a remedy against CCl4-induced hepatotoxicity.

4.1. Study limitations

There are a number of limitations in this study. The first limitation of the study is that the findings in the current work are based on animal study (in vivo), and hence, they may not be directly applicable in clinical practice. The second limitation is that the present study is a proof of concept and that the results in this study will form the basis for clinical testing. Another drawback is that the results were based on one animal species, rats only, and that comparative studies with other animal species should also be pursued. Also, the study did not attend to further investigate all indicators of liver fibrosis.

In conclusion, the results showed that blueberry and cranberry extracts have a protective effect against subacute and subchronic

CCL4-induced hepatotoxicity in rats. The results confirm that cranberry and blueberry extracts can suppress fibrosis, inflammation and oxidative stress in rats with liver damage and prevent CCL4-induced liver injury, as well as have good antifibrotic and hepatoprotective function. However, the establishment of the most effective combinations and concentrations, and their clinical use remain insufficiently studied and require further research in order to determine the maximum hepatoprotective and antifibrotic effect.

Author contribution statement

Shynggys Sergazy, Zarina Shulgau, Yevgeniy Kamyshanskiy: Performed the experiments.

Elena Krivyh, Alexander Gulyayev: Analyzed and interpreted the data.

Zhaxybay Zhumadilov: Contributed reagents, materials, analysis tools or data.

Mohamad Aljofan: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

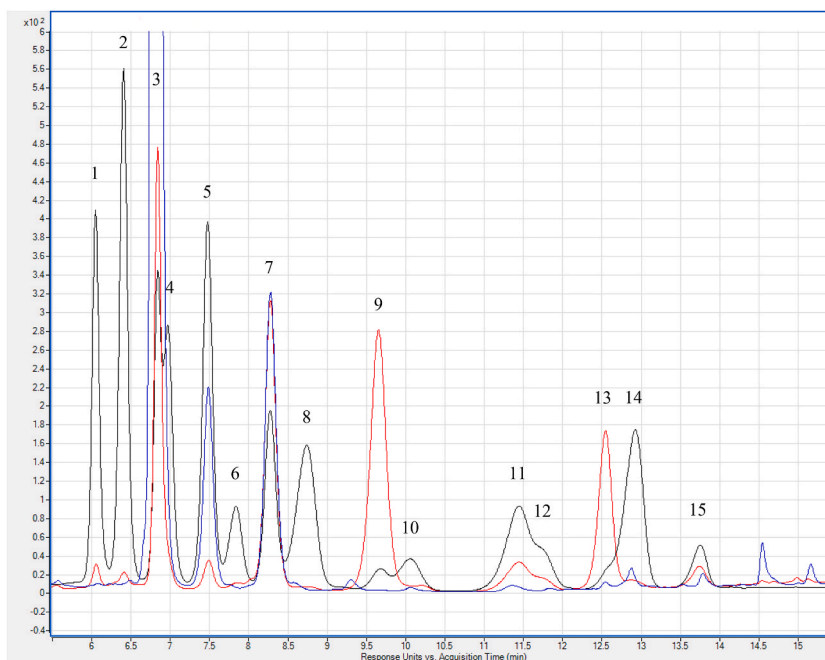
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 1

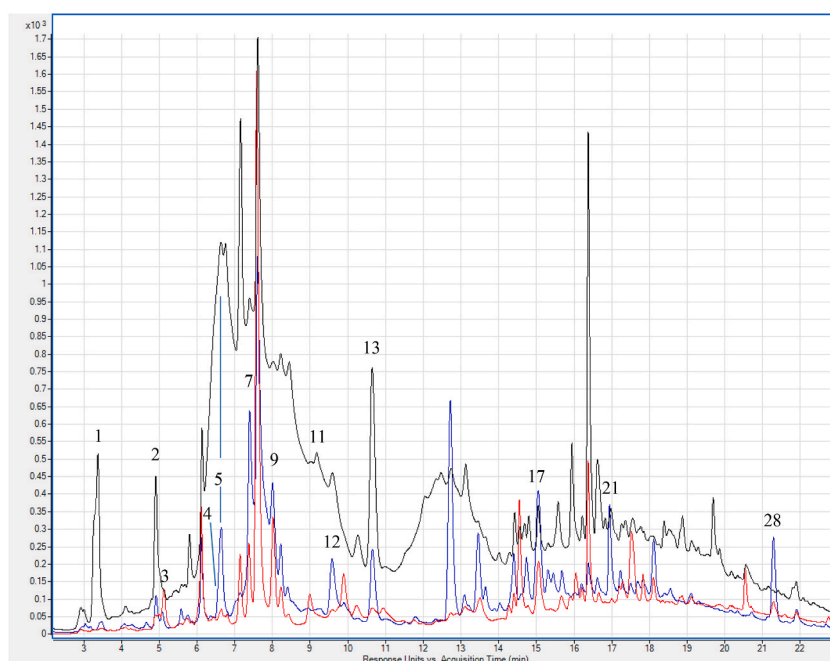


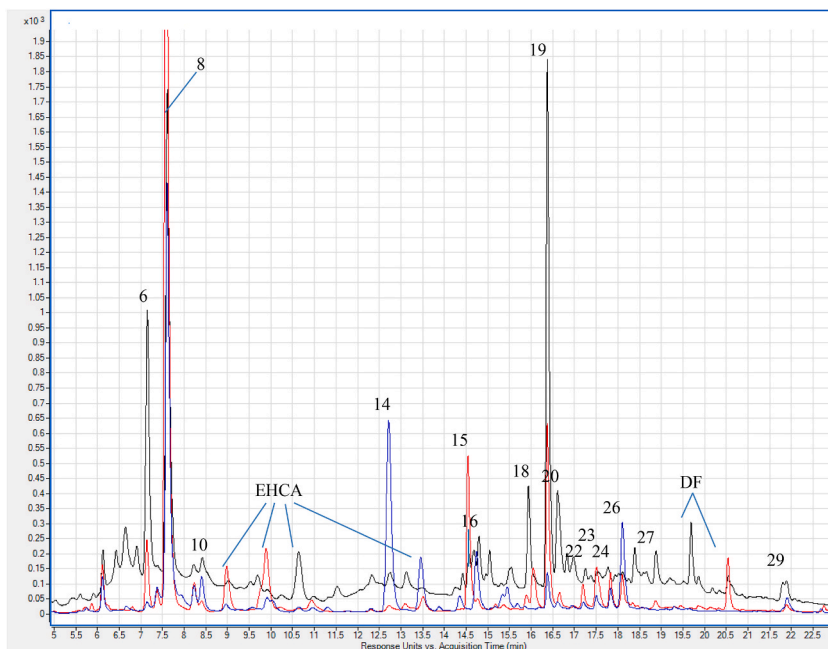
Appendix-Figure 1. Anthocyanins in chromatograms of extracts of cranberry (red line), lingonberry (blue line) and blueberry (black line). λ 520 nm. The numbering corresponds to Table 1.

Appendix Table 1

Composition of anthocyanins in berry extracts, mg/l.

N ^o	anthocyanin	cranberry	lingonberry	blueberry
1	Delphinidin-3-O-galactoside	6,3		1268
2	Delphinidin-3-O-glucoside st	3,8		1985
3	Cyanidin-3-O-galactoside	115	818	945
4	Delphinidin-3-O-arabinoside			1043
5	Cyanidin-3-O-glucoside	9,2	64	1495
6	Petunidin 3-O-galactoside			379
7	Cyanidin-3-O-arabinoside	111	109	817
8	Petunidin-3-O-glucoside st	2,0		971
9	Peonidin-3-O-galactoside	135		129
10	Petunidin-3-O-arabinoside		1,3	239
11	Peonidin-3-O-glucoside st	23	4,0	777
12	Malvidin-3-O-galactoside		1,5	263
13	Peonidin-3-O-arabinoside	70	1,7	98
14	Malvidin-3-O-glucoside st	3,5	7,0	1173
15	Malvidin-3-O-arabinoside	10	3,0	259
	Bcero	489	1026	11,842

st — identification confirmed by analytical standard.**Appendix Fig. 2.** Hydroxybenzoic acids and flavan-3-ols in chromatograms of extracts of cranberry (red line), lingonberry (blue line) and bilberry (black line). λ 280 nm. The numbering corresponds to appendix Table 2.



Appendix Fig. 3. Hydroxycinnamic acids and flavonols in chromatograms of extracts of cranberry (red line), lingonberry (blue line) and bilberry (black line). λ 325 nm. The numbering corresponds to Table 2. EHCA - esters of hydroxycinnamic acids. DF - are derivatives of flavonols.

Appendix Table 2

Composition of phenolic compounds in berry extracts, mg/l.

N ^o	Phenolic compound	cranberry	lingonberry	blueberry
1	Gallic acid			173
2	Protocatechuic acid	10	26	118
3	Tyrosol	6,4		
4	4-Hydroxybenzoic acid		~39	
5	Procyanidin B 1	26	~195	49
6	3- <i>p</i> -Coumaroylquinic acid	30		130
7	(+)-Catechin	113	438	42
8	Chlorogenic acid (5-Caffeoylquinic acid)	533	240	342
9	Procyanidin	179	243	16
10	<i>trans</i> -Caffeic acid	4,3	13	4,7
11	Syringic acid			10
12	Procyanidin B 2	13	233	42
13	(-)-Epicatechin	20	222	~700
14	<i>trans-p</i> -Coumaric acid	3,7	119	10
15	Myricetin-glycoside	168		33
16	<i>trans</i> -Ferulic acid	3,9	24	
17	Procyanidin	152	327	
18	Quercetin-3-glucuronide			130
19	Quercetin-3-galactoside	186	36	606
20	Quercetin 3-glucoside	15	6,2	156
21	Procyanidin		178	
22	Quercetin 3-xyloside	24	7,9	
23	Quercetin 3-arabinopyranoside	55	16	
24	Quercetin 3-arabinofuranoside	32	20	
25	Kaempferol 3-glucoside			6,6
26	Quercetin-3-rhamnoside (quercitrin)	25	83	7,4
27	Myricetin	9,2		43
28	Procyanidin	32	203	
29	Quercetin	9,4	25	26
30	Kaempferol		0,7	
	Hydroxybenzoic acids	17	65	301
	Hydroxycinnamic acids	575	397	487
	Flavonols	524	195	1007
	Flavan-3-ols	535	2039	890
	Total:	1651	2696	2685

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