*https://doi.org/10.31925/farmacia.2022.4.11* **ORIGINAL ARTICLE**

# EFFICACY OF NATURAL POLYPHENOLIC COMPOUNDS FROM BILBERRY AND BLUEBERRY ON THE METABOLIC ALTERATIONS INDUCED BY STREPTOZOTOCIN IN RATS

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**Abstract**

Chronic hyperglycaemia is associated with dysfunction of various organs. We have previously reported that *Vaccinium myrtillus* extracts are capable of reducing hyperglycaemia, modulating the secretion of insulin and preventing the development of cataract. The primary objective of this study was to assess the effects of orally administered high polyphenolic content extracts on diabetes-induced complications in rats. After 3 months of treatment with *Vaccinium myrtillus* and *Vaccinium corymbosum* extracts, hepatic enzymes, serum creatinine and lipids were measured, and histopathological alterations were evaluated in Wistar rats with streptozotocin-induced diabetes. Histopathological staining revealed that only minor signs of liver impairment were noticed in the *Vaccinium myrtillus* leaves group, compared with *the Vaccinium corymbosum* leaves and fruits treated group, where severe fibrosis was noticed. The liver histopathological results are correlated with AST levels. Moreover, some histopathological alterations of the kidneys were observed in the groups treated with *V. corymbosum* extracts, compared with *V. myrtillus* treated groups, where only minor modifications were noticed. Extracts with high polyphenolic content from *Vaccinium myrtillus* are capable of delaying and/or preventing the complications of induced diabetes, like renal and liver impairment.

**Rezumat**

Hiperglicemia cronică este asociată cu disfuncția diferitelor organe. Prin cercetările anterioare am demonstrat că extractele de *Vaccinium myrtillus* sunt capabile să reducă hiperglicemia, să moduleze secreția de insulină și să prevină dezvoltarea cataractei. Obiectivul principal al acestui studiu a fost de a evalua efectele extractelor cu conținut ridicat de compuși polifenolici, administrate oral, asupra complicațiilor induse de diabet la șobolani. După 3 luni de tratament cu extracte de *Vaccinium myrtillus* și *Vaccinium corymbosum*, s-au determinat enzimele hepatice, creatinina serică, lipidele serice, împreună cu modificările histopatologice, la șobolani Wistar cu diabet indus de streptozotocină. Colorația histopatologică a evidențiat că doar semne minore de afectare hepatică în grupul tratat cu extract de frunze de *Vaccinium myrtillus*, în comparație cu grupul tratat cu extract din frunze și fructe de *Vaccinium corymbosum*, unde a fost observată fibroză severă. Rezultatele histopatologice sunt corelate cu nivelul de AST. Mai mult, s-au observat unele modificări histopatologice ale rinichilor în cazul loturilor tratate cu extracte de *V. corymbosum*, comparativ cu loturile tratate cu *V. myrtillus*, unde doar modificări minore au fost observate. Extractele cu conținut ridicat de polifenolici din *Vaccinium myrtillus* sunt capabile să întârzie și/sau să prevină complicațiile diabetului indus, cum ar fi insuficiența renală și hepatică.

**Keywords**: *Vaccinium*, diabetes, bilberry, blueberry

## Introduction

Diabetes is a serious pathology affecting millions of people worldwide. The number of diabetic patients is increasing at an alarming rate, and according to World Health Organization, over 422 million peoples suffer from diabetes [26]. Lately, with the appearance of the new coronavirus, diabetes can worsen the outcome of the disease [7].

This increment is determining researchers to evaluate complementary treatments that have additive effects with the long-term maintenance of pharmacological therapy. The aim of pharmacological and phyto-pharmacological therapy is to achieve an improved quality of life and reduced mortality. With the progress of diabetes, due to the non-enzymatic formation of advanced glycosylated end products (AGE's), risks of developing complications are increased in the non-insulin dependent tissues. The most severe microvascular complications are nephropathy, neuropathy and retino-pathy. The search for natural compounds that can maintain a stable glucose level and prevent diabetic-related complications opens new frontiers in the natural product domain. *Vaccinium* species are known to have a complex phytochemical profile, and *in vitro* studies have demonstrated that the extracts are capable of modulating different enzymes involved in this pathology [2, 17].

Bilberry (*Vaccinium myrtillus*) leaves are used in Romanian traditional medicine based on empirical proofs. Blueberry (*Vaccinium corymbosum*) is cultivated exclusively for its fruits [11]. The leaves are under-explored sources of natural antioxidants due to their high polyphenolic content but are a waste product at this moment [21].

Various compounds found in bilberry leaves, like chlorogenic acid, for example, exert some effects on diabetes. Still, it seems that rather the phytocomplex has the most remarkable action in ameliorating the symptoms related to the metabolic disease [9, 16]. It appears that the various polyphenolic compounds act in synergy, modulating different targets. Considering this theory, the first premise of our study was to evaluate the effects of different extracts, alone and in combination. We have previously demonstrated that the *Vaccinium myrtillus* extracts are capable of reducing hyperglycaemia, so we further intended to evaluate the other possible metabolic effects. The second premise was to evaluate the effects of two extracts obtained from a cultured specie in order to observe if this sustainable herbal material could be further exploited.

## Materials and Methods

*Sample collection*

*Vaccinium corymbosum* fruits and leaves were collected from a culture in Mureș County, Romania. *Vaccinium myrtillus* leaves and fruits were collected from Călimani Mountains, Mureș County, Romania. The leaves were air dried in laboratory conditions, and the fruits were immediately frozen at -20°C. For each sample, a voucher was added to the herbal drugs collection of the Department of Pharmacognosy and Phytotherapy from our University: RVM15 (*Vaccinium myrtillus* leaves), RVC15 (*Vaccinium corymbosum* leaves), RVM202 (*Vaccinium myrtillus* fruits) and RVC202 (*Vaccinium corymbosum* fruits). All chemicals and reagents were of certified analytical grade.

*Extracts preparation*

Polyphenolic rich extracts were obtained by ultrasound-assisted extraction. The plant material was extracted with 50% ethanol at a final concentration of 5% for dried leaves and 15% for fresh fruits. After filtration, the extracts were concentrated under reduced pressure. This procedure was repeated 3 times, and the resulting concentrated extracts were combined.

*Quantitative determination of total polyphenolic content, flavonoids and tannins*

Total polyphenolic content was determined with the Folin-Ciocâlteu reagent as previously described [23]. Total flavonoids were determined from the leaves, following the method described in the Romanian Pharmacopoeia X$^{th}$ edition, under the *Cynarae folium* monography [27]. Rutin was used as standard for the calibration curve in a concentration range of 2 - 20 mg/L. The results were expressed as g rutin equivalents *per* 100 g dry weight (mg RE *per* 100 g DW). The determination of tannins from the leaves was carried out according to the method described in the European Pharmacopoeia 7.0, and the absorbance was measured at 760 nm on a Specord 210 spectrophotometer. The percentage content of tannins was expressed as mg pyrogallol/100 g dry weight for leaves or 100 g fresh weight for fruits. The percent content of anthocyanins was determined according to the method described in the European Pharmacopoeia 7$^{th}$ Edition at the monograph of bilberry fruit, fresh [28].

*In vivo experimental design*

*Experimental animals*

The research was conducted according to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, and the experimental protocol was approved by the Ethics committee of the "George Emil Palade" University of Medicine, Pharmacy, Science and Technology of Târgu-Mureș, Romania. The adult female Wistar rats were provided by the University's Department of Experimental Animal Facilities. The animals were housed alone in standard propylene cages under control conditions (temperature: 23 ± 2°C, humidity: 55 ± 5%, 12-hour light/dark cycle). Commercial diet and tap water were *ad libitum* during the experiment. Before the beginning of the experiment, the animals were acclimatized for 1 week.

*Diabetes induction*

Diabetes was induced by a single intraperitoneal injection with a freshly prepared solution of streptozotocin at a dose of 60 mg/kg body weight. After the injection, animals had free access to water and pellets to prevent hypoglycaemia caused by the massive release of insulin from pancreatic cells [19]. After 48 hours, glucose level was measured, and the rats with at least 250 mg/dL of blood glucose were considered diabetic and included in the present study.

*Experimental treatments*

Rats were randomly divided into 7 groups, each containing 8 animals: NC – non-diabetic control group, diabetic control group, PC – positive control group (500 mg/kg metformin), VML – diabetic rats treated with *Vaccinium myrtillus* extract, VMLF – diabetic rats treated with *Vaccinium myrtillus* leaves and fruits extract, VCL – diabetic rats treated with *Vaccinium corymbosum* leaves extract, VCLF – diabetic rats treated with *Vaccinium corymbosum* leaves and fruits extract. Treatment was administered through gavage for eight weeks. In the last week of the present experiment, the volume of excreted urine was measured for 24 hours. At the end of the study, rats were sacrificed, and the liver and kidneys were collected, weighed and immersed in 10% formalin for histopathological analysis. Blood was collected, and the serum was separated and frozen at -70°C until analysis.

*Biochemical determinations*

Creatinine, total cholesterol, triglycerides, ALT and AST levels were determined using a fully-automated Cobas Integra 400 Plus analyser. For the total cholesterol CHOL2 reagent (Colesterol Roche Diagnostics, GmbH, Mannhaim, Germany) was used and the measurement range was 3.87 - 800 mg/dL. For triglycerides, TRIGL reagent (TRIGL Roche Diagnostics, GmbH, Mannhaim, Germany) was used and the measurement range was 8.85 - 885 mg/dL. For ALT and AST a standardized kinetic method (International Federation for Clinical Chemistry – IFCC) with pyridoxal phosphate was used, and the measurement range was 2.0 - 700 U/L. Creatinine level was assessed using CREJ2 reagent (Creatinine Jafee gen.2, Roche Diagnostics, GmbH, Mannhaim, Germany) and the measurement range was 0.2 - 15 mg/dL.

*Histopathological analysis*

After the sacrification of the animals, the liver and kidneys were weighed, and the organs were fixed in 10% formalin until paraffin embedding. Paraffin blocks were cut using a microtome, and the sections were stained using haematoxylin and eosin (HE), Periodic acid-Schiff reagent (PAS) and Verhoeff's van Gieson (VvG) [6]. Histopathological examination was performed using an Olympus microscope BX46, coupled with an Olympus SC100, U-CAMD 3 camera, Tokyo, Japan.

*Statistical analysis*

GraphPad Prism 9 was used for the statistical analysis. All data are presented as mean ± standard deviation (SD). Results were tested by Student *t*-test or ANOVA followed by Tukey's post-hoc comparative test.

## Results and Discussion

*Quantitative determination of total polyphenolic content, flavonoids and tannins*

Our results (Table I) indicated that *Vaccinium myrtillus* leaves contain a high percentage of polyphenolic compounds, including tannins and flavonoids. Similar results have been obtained from fruits (Table II), and there is a significant difference ($p < 0.05$) between the polyphenolic composition of the analysed fruits. *Vaccinium myrtillus* fruits contain significantly higher concentrations of tannins and flavonoids compared with *Vaccinium corymbosum* fruits, although the total polyphenolic content is not significantly different.

**Table I**

Content of determined phenols in blueberry and bilberry leaves

| | **Tannins (g pyrogallol/ 100 g DW)** | **Flavonoids (g RE/100 g DW)** | **Total polyphenols (g GAE/100 g)** |
|---|---|---|---|
| ***Vaccinium myrtillus* leaves** | $7.68 \pm 0.29^a$ | $1.14 \pm 0.127^a$ | $14.82 \pm 0.76$ |
| ***Vaccinium corymbosum* leaves** | $5.7 \pm 1.58^b$ | $0.88 \pm 0.094^b$ | $12.71 \pm 0.42$ |

Values in the same columns with different superscript letters are significantly different ($p < 0.05$)

**Table II**

Content of determined phenols in blueberry and bilberry fruits

| | **Total polyphenols* (g GAE/100 g)** | **Anthocyanins (g/100 g)** |
|---|---|---|
| ***Vaccinium myrtillus* fruits** | $0.56 \pm 0.005^a$ | $0.52 \pm 0.019^a$ |
| ***Vaccinium corymbosum* fruits** | $0.26 \pm 0.027^b$ | $0.11 \pm 0{,}01^b$ |

Values in the same columns with different superscript letters are significantly different ($p < 0.05$)

Spectrophotometric determinations were carried out for both types of leaves and both types of fruits in order to quantify the main pharmacological active compounds. The results obtained on the determination of polyphenolic compounds from fruits are in accordance with other published data and have shown that these products contain high concentrations of polyphenolic compounds [3, 12].

*In vivo study*

*Biochemical determinations*

Treatment with *Vaccinium* extracts was found to decrease AST and ALT levels in all, but one group compared to the DC group. The group treated with blueberry leaves extract (VCL group) had a notably elevated AST level. In insulin resistance, hepatic dysfunction is a common phenomenon. Hepatic enzymes were significantly higher in the diabetic control group compared

to the normal control group. Aminotransferases catalyse the transfer of the amino groups from aspartate or alanine, forming oxaloacetic acid and pyruvic acid, respectively. Oxaloacetic acid and pyruvic acid suffer an enzymatic reduction to malate and lactate. The reaction is coupled to the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide ($NAD^+$) [8]. Hepatic cytolysis, as an effect of glucose homeostasis impairment, leads to high values of aminotransferases. Alanine aminotransferase is the specific marker for hepatic alterations due to its distribution mostly in the liver. Therefore, these results reveal an effectively protective role of *Vaccinium myrtillus* leaf extract against hepatic alterations. The mechanisms underlying the beneficial effects of *Vaccinium myrtillus* polyphenols could be similar to that of silymarin that has a hepatoprotective and antioxidant effect by enhancing the hepatic glutathione [22].

As seen in Table II, there are no statistically significant differences ($p < 0.05$) between the triglyceride levels determined in the seven groups, but a higher level of TG in the non-treated group 2 can be observed. However, due to the close values and the large difference recorded in the same group, a pertinent conclusion cannot be drawn regarding the effect of extracts on lipid metabolism. Dyslipidaemia is a feature of type 2 diabetes. Although frequently encountered in type 1 diabetes, the lipid values are normal or near normal, macro- and micro-vascular complications and acute myocardial infarction are common [14]. It appears to be a contradiction in the literature regarding hypertriglyceridemia in type 1 diabetes. In some animal model experiments, it was observed that low insulin secretion reduced endogenous synthesis and TG secretion in the liver, resulting in normal TG values; other studies suggest, however, that in insulin-deficient diabetes, when there is poor glucose control, hypertriglyceridemia occurs due to the body's inability to take TG from food and the peripheral lipolysis is affected, due to the inadequate insulin/glucagon ratio [15, 24].

The urine volume was significantly higher in DC, PC and VMLF groups compared to the normal NC group (Figure 1). Although VML, VCL and VCLF groups had a higher urine excretion compared to normal control, there are no significant statistical differences. In a hyperglycaemic state, polyuria appears due to a glucose-induced osmotic diuresis. The presence of polyuria was noticed from the first week after the induction of diabetes. Along with the progress of the experiment, changes regarding the urine output were noticed. A higher reduction of urine output was noticed in the groups treated with extracts from the leaves of *Vaccinium myrtillus* and *Vaccinium corymbosum*.

Diabetogenic action of streptozotocin appears due to the alkylation of DNA, leading to the activation of ADP-ribosylation, causing the depletion of $NAD^+$ and ATP. This step produces a substrate for the reactions catalysed by xanthine oxidase and the formation of reactive oxygen species [18].

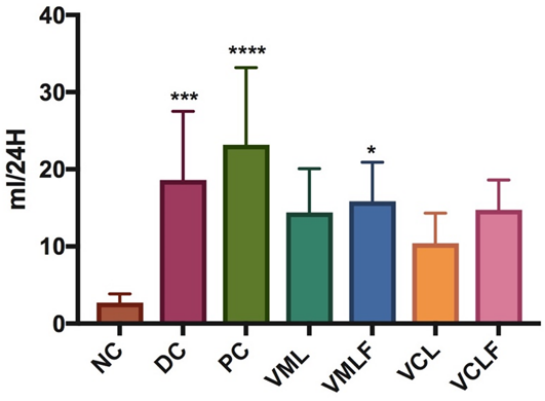


**Figure 1.**

The volume of urine excreted in 24 hours

NC – non-diabetic control group; DC – diabetic control group; PC – positive control group treated with metformin; VML – diabetic group treated with *Vaccinium myrtillus* leaves extract; VMLF – diabetic group treated with *Vaccinium myrtillus* leaves and fruits extract; VCL – diabetic group treated with *Vaccinium corymbosum* leaves extract; VCLF – diabetic group treated with *Vaccinium corymbosum* leaves and fruits extract. Values are means ± standard deviation. * Significantly different from NC ($p < 0.05$); *** significantly different from NC ($p < 0.001$); **** significantly different from NC ($p < 0.0001$).

In our previous study, we confirmed that *Vaccinium* extracts are capable of reducing high blood glucose levels in diabetic rats and also enhancing the secretion of insulin [20]. The aim of this study was to further evaluate the effects of four extracts on hepatic enzymes, serum lipids and creatinine in correlation with the histopathological alterations caused by the induction of diabetes with streptozotocin in rodents. Metformin was chosen as the treatment for the control group due to the particularities of its action, like the extrapancreatic mechanism of action, low risk of producing hypoglycaemia, antioxidant effects, etc. According to previously published research, the oral dose of metformin was 500 mg/kg, as it has been shown to have stronger clinical effectiveness compared to low doses of metformin. Because of the high tannin content, the dosage protocol for the *in vivo* study was limited. A high tannin intake could lead to constipation and/or to a lower absorption of iron, which can cause anaemia. Creatinine is an indicator of renal failure, which is increased if there is renal impairment. No significant differences were observed in creatinine levels. It can be noticed, however, that in DC and VCL groups, serum creatinine is slightly higher than in the other groups. Although no significant differences were observed regarding the creatinine level, the higher levels noticed in the DC group are probably due to the progression to diabetic nephropathy. Still, this complication could not be confirmed by histopathological analysis of the kidney.

**Table III**

Biochemical parameters

| Group | AST (U/L) | ALT (U/L) | Col (mg/dL) | TG (mg/dL) | Creatinine (mg/dL) |
|---|---|---|---|---|---|
| **NC** | 190 ± 76.8$^{a}$ | 68.1 ± 23.2$^{b}$ | 73.8 ± 27.7$^{ab}$ | 101.7 ± 40.4 | 0.46 ± 0.06 |
| **DC** | 493.6 ± 123.9$^{b}$ | 163.5 ± 69.3$^{a}$ | 54.9 ± 16.8$^{a}$ | 136.3 ± 58.2 | 0.51 ± 0.1 |
| **PC** | 400.8± 187.5$^{ab}$ | 94.4 ± 35.7$^{ab}$ | 68.9 ± 11.7$^{ab}$ | 114.7 ± 50.4 | 0.54 ± 0.13 |
| **VML** | 313.2 ± 114$^{ab}$ | 81.5 ± 41.1$^{b}$ | 56 ± 15.4$^{a}$ | 105.2 ± 46.2 | 0.47 ± 0.13 |
| **VMLF** | 381.7± 197.6$^{ab}$ | 105.6 ± 51.3$^{ab}$ | 60 ± 10.7$^{ab}$ | 107.7 ± 41.7 | 0.44 ± 0.06 |
| **VCL** | 547 ± 218.8$^{b}$ | 114.7 ±30.8$^{ab}$ | 87.7 ± 15.9$^{b}$ | 92.3 ± 17.8 | 0.52 ± 0.06 |
| **VCLF** | 318.5 ± 99.4$^{ab}$ | 104.1 ± 58.9$^{ab}$ | 60 ± 17.4$^{ab}$ | 112.3 ± 43.7 | 0.42 ± 0.13 |

NC – non-diabetic control group; DC – diabetic control group; PC – positive control group treated with metformin; VML – diabetic group treated with *Vaccinium myrtillus* leaves extract; VMLF – diabetic group treated with *Vaccinium myrtillus* leaves and fruits extract; VCL – diabetic group treated with *Vaccinium corymbosum* leaves extract; VCLF – diabetic group treated with *Vaccinium corymbosum* leaves and fruits extract. Values in the same column with different superscript letters are significantly different ($p < 0.05$)

*Histopathological analysis*

The organs were weighed as soon as they were removed. Although there are no statistically significant differences, it has been observed in the diabetic group (DC) that both kidneys and the liver had a lower weight compared to the control group (NC). Histopathology of the kidney (Figure 2) in the normal control group revealed a normal structure with no pathological modifications: no signs of inflammation or congestion. In the DC group, the cell base membrane was thickened.

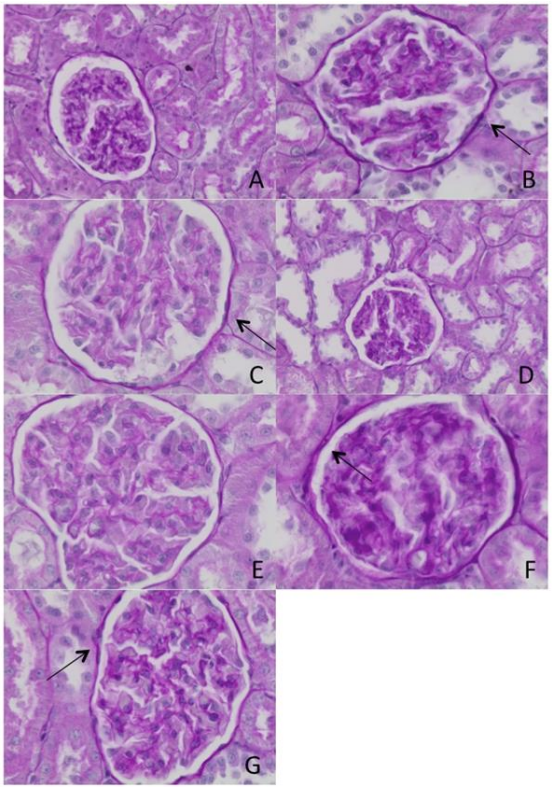


**Figure 2.**

Histopathological analysis of the kidney (PAS staining)

Arrows indicate thickened glomerular basement membrane (GBM). A: NC group, normal histology, 20x; B: DC group, most glomeruli had thickened GBM, 40x; C: PC group, thickened GBM of glomeruli, 40x; D: VML group, normal histology, 20x; E: VMLF group, areas with thickened GBM, 40x; F: VCL group, severe thickened GBM, 40x; G: VCLF group, areas with thickened GBM, 40x.

Hyperglycaemia produced mild damage to renal functions. Only a thickening in the basal membrane of the glomeruli was observed and a slight hyalinization in some of the diabetic groups. The group treated with *Vaccinium myrtillus* leaf extract showed a minor thickening of the GBM, with no other pathological alterations. The same response was observed in the group treated with *Vaccinium myrtillus* leaf and fruit extract. The two groups treated with *Vaccinium corymbosum* extracts had a more thickened basal membrane.

The liver section (Figure 3) from normal control rats showed normal hepatic architecture and the absence of fibrosis. Histopathological analysis of the liver from diabetic control rats showed signs of hepatic alterations, like severe inflammatory cell infiltration, mononuclear and polymorphonuclear infiltrate, inflammatory infiltration in sinusoids and increased fibrosis. In the group treated with metformin (PC), fewer alterations were noticed compared to the DC group. In the VML group, there were only minor changes; the liver parenchyma had a normal aspect with few areas with adipose degeneration. Peri-portal inflammatory infiltration was noticed in the VMLF group. In the VCL group, bile duct hyperplasia was evident, with no other pathological changes. In the VCLF group, changes like adipose degenerations, dilated sinusoids, Kupffer cells, inflammatory infiltrate and severe fibrosis were noticed.

Hyperlipaemia causes fatty liver formation. Streptozotocin-induced diabetes, which causes a hypoinsulinic state, leads to pathologic lesions in the liver, similar to the changes that occur in diabetic patients whose blood glucose is poorly controlled [25]. Sinusoids were dilated, and an increased number of Kupffer cells were observed. Activation of Kupffer cells is usually a marker of liver injury.

Dietary polyphenols are recommended for a series of effects in the symptoms caused by metabolic disease. According to the scientific literature, anthocyanins have been found to have beneficial effects on the lipid profile. There are several mechanisms incriminated to be responsible for these effects. Their antioxidant activity could be responsible for the interference in the

oxidation of lipoproteins mediated by the free radicals [4, 5].

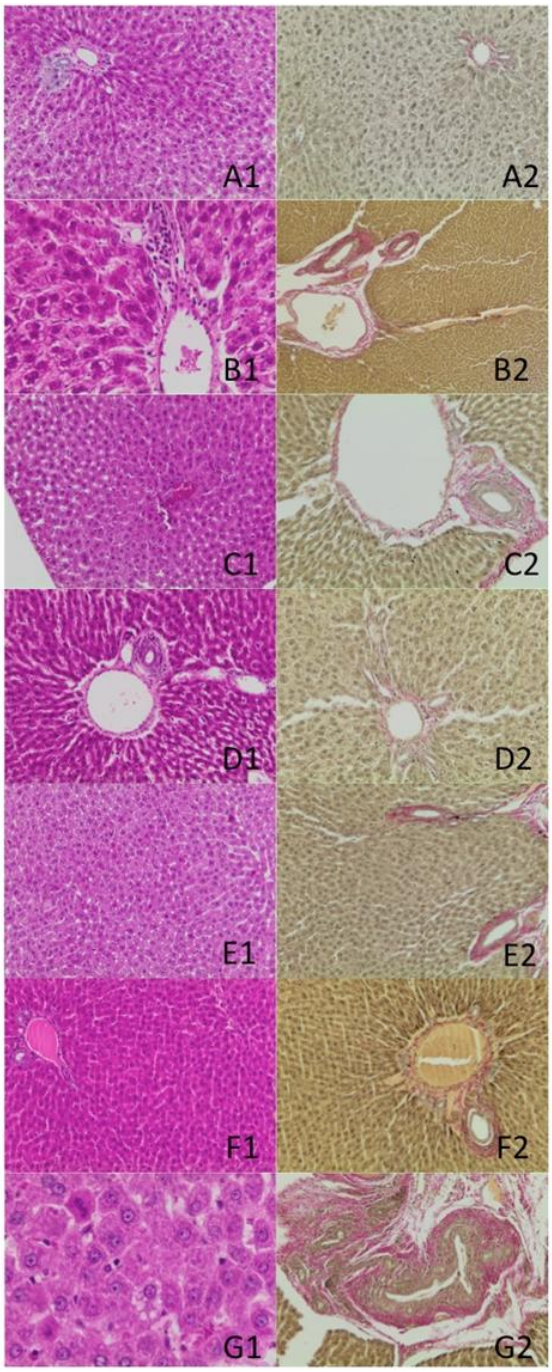


**Figure 3.**
Histopathological analysis of the liver
The sections from the first column (1) are stained with H&E, and the sections from the second column are stained with VvG. A1, A2: NC group, no pathological changes; B1: DC group, severe inflammatory cell infiltration, mononuclear and polymorphonuclear infiltrate; B2: DC group, peri canalicular fibrosis; C1: PC group, stasis; C2: PC group, fibrosis; D1, D2: VML group, normal liver histology with portal triad; E1: VMLF group, fat degeneration; E2: VMLF group, fibrosis; F1: VCL group, normal parenchyma; F2: VCL group, bile duct hyperplasia; G1: VCLF group, dilated sinusoids with Kupffer cells; G2: VCLF group, severe fibrosis

Extracts with high polyphenolic content from bilberry can be further used in functional foods and beverages that can prevent and ameliorate different symptoms in metabolic diseases.

## Conclusions

The present study was designed to assess the possible implications of blueberry and bilberry leaves and fruit extracts on the liver and kidney function of diabetic rats. Bilberry leaves can successfully prevent a series of diabetic complications, like liver injury and nephropathy, and are also involved in lipid metabolism. Finally, we can conclude that the leaf and fruit extracts of *Vaccinium corymbosum* did not have the beneficial effects sought and reported in the literature. This preclinical study highlights the importance of future studies focusing on *Vaccinium* species for applications in drug development.

## Conflict of interest

The authors declare no conflict of interest.

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