The effect of the methanol extract of Caucasian whortleberry on glucose, oxidative biomarkers, cholesterol and HDL in diabetic male rats

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Abstract

Introduction:
Vaccinium arctostaphylos is a medicinal plant used for the treatment of diabetes. The present study was conducted to investigate the effect of the methanol extract of Caucasian whortleberry on glucose, oxidative biomarkers, HDL and cholesterol.

Materials and methods:
The present experimental study was conducted on 24 male Wistar rats divided into a healthy control group, a diabetic control group and two diabetic trial groups treated with 150 and 250 mg/kg of body weight doses of the methanol extract of Caucasian whortleberry. After inducing diabetes in the rats, the effect of 30 days of intraperitoneal injection of the extract was examined on their blood biochemical markers.

Results:
Comparing the two diabetic trial groups treated with Caucasian whortleberry extract revealed the 250 mg/kg dose to be the most effective concentration for reducing glucose, increasing HDL, increasing paraxonase activity, reducing malondialdehyde and reducing body weight, while the 150mg/kg dose of the extract was the most effective for reducing cholesterol and protein carbonyl (P<0.05).

Conclusion:
The present study showed that the methanol extract of Caucasian whortleberry can reduce the adverse effects of diabetes on many biochemical markers and thus boasts medicinal properties.

Keywords: Glucose, Cholesterol, Malondialdehyde, Protein Carbonyl, Paraoxonase

Introduction
Diabetes is known as one of the most common chronic diseases worldwide and a leading cause of death. The prevalence of the disease is increasing, such that the number of people living with diabetes is expected to rise from 171 million in 2000 to 366 million by 2030 (1). Pancreatic beta cells dysfunction and insulin resistance play important roles in the development of the disease (2). Diabetes is considered as the most important risk factor for many disorders such as nephropathy, retinopathy, neuropathy and cardiovascular diseases (3). The prevalence of cardiovascular diseases in diabetics is 2-4 times more than that in healthy individuals (4).

In recent years, the relationship between deadly complications of diabetes and increased glucose levels, blood lipid profile, reduced antioxidant defense, resulting in an increase in oxidative reactions in the body were examined. Studies indicate that increased formation of free radicals due to hypoglycemia has a
major role in the pathogenesis and progression of diabetic complications (5). Free radicals, along with increased amounts of glucose and lipoproteins in the blood exacerbate the process of peroxidation or glycosylation. This process leads to toxic reactions in endothelial cells and the accumulation of LDL causes atherosclerosis progression as a fatal complication of diabetes (6). Oxidative stress is as a result of an imbalance between the production of free radicals and reactive oxygen species on the one hand, and antioxidant defense system on the other, which may play an important role in pathophysiology of insulin resistance, diabetes and its complications through increased oxidative damage, inflammation, apoptosis and delayed complications of diabetes (7). Carbonylation is an irreversible deformation by oxidative stress, which often leads to dysfunction and changes in the biological activity of proteins and oxidative formation of protein carbonyl (PC) (8). LDL oxidation is another complication of increased free radicals, which is known as a key step to begin the process of atherosclerosis. Paraoxonase 1 is an esterase with an antioxidant effect bound to HDL that fights oxidative damage of lipids through toxic oxidized lipid metabolism of LDL and HDL. This enzyme prevents the formation of oxidized LDL and deactivates oxidized LDL-derived phospholipids and justifies its role in cardiovascular diseases. Diabetes, thyroid diseases, metabolic syndrome, renal failure and aging are associated with reduced paraoxonase activity (9,10). Several studies have investigated the relationship between high blood sugar and lipid peroxidation. Passaglia et al. (2004) showed that malondialdehyde is significantly increased in patients with type II diabetes (11). It is formed during the biosynthesis of prostaglandins in the metabolism of arachidonic acid and then reacts with amino acids of proteins and other biomolecules in cells and creates mutagenic compounds that cause cancer (12). Due to various complications of diabetes, it is essential to find a treatment. The use of medicinal plants for the treatment of diabetes has long been common and today is considered an alternative (13). The efficiency of these plants in lowering blood sugar and given that they are welcomed by people have facilitated the usage of these plants in the society. However, the rational administration of medicinal plants requires accurate knowledge of their mechanism (14). Vaccinium macrocarpon is one of the plants used in traditional medicine for its anti-diabetic effects. Numerous studies have been conducted on the medicinal properties of two species, with scientific names Vaccinium myrtillus and Vaccinium arctostaphylos (15). In Iran, only the second species grows in the highlands of Ardabil province. Empirical evidence suggests the effect of the plant in lowering blood sugar in normal animals and alloxan-induced or streptozotocin-induced diabetic rats (16-17). The anti-diabetic effect of Vaccinium macrocarpon was confirmed in human studies (18). The mechanisms proposed so far for reducing blood sugar include: inhibition of glucose absorption from the intestine, increased cellular glucose uptake (19) and inhibition of gluconeogenesis in the liver (20). Anthocyanins are the most important components of the plant. Evaluations of metabolites of the plant by Nickavar et al. (2003) showed that ripe fruits of Vaccinium macrocarpon contain three main anthocyanins and thus the plant can be considered as an important medicinal plant (21). Blood sugar is increased in diabetic rats due to streptozotocin-induced insulin-deficiency (22). Streptozotocin is synthesized by streptomyces acromogens (23), impairs glucose oxidation, and reduces the synthesis and secretion of insulin. Streptozotocin enters pancreatic beta cells with GLUT2 and causes tissue damage. The toxic effects of streptozotocin in pancreatic beta-cell occur
due to changes in cellular DNA. Recent experiments have shown that the prime reason for cell destruction and apoptosis is DNA alkylation (24-25). This impairment may increase serum triglyceride levels because insulin is effective in lipid metabolism. Given the inverse relationship of TG concentration and HDL, decreased HDL levels and increased LDL are expected in diabetic rats (22). HDL is a high-density lipoprotein which plays an important role in cardiovascular diseases and its low concentration is one of main risk factors for these diseases (26). In people with type II diabetes HDL is reduced and it can protect LDL against oxidative damage and prevent the creation of oxidized LDL (27-28). In the study of Lee et al. (2008) Vaccinium macrocarpon was used for patients with type II diabetes at a dose of 1500 mg daily for 12 weeks and the results showed reduced LDL and total cholesterol and increased HDL levels (29). This study aimed to determine the effect of methanol extract of Vaccinium macrocarpon on oxidative biomarkers such as malondialdehyde, paraoxonase activity, and protein carbonyl as well a glucose levels, LDL and cholesterol in male diabetic rats.

Materials and methods
In this experimental study, Vaccinium macrocarpon fruit was first identified by an expert from Agricultural Research Center, Isfahan. Then, the fruit was milled, soaked in ethanol 70% to be extracted. Twenty-four adult male Wistar rats (Rattus norvegicus allivias) weighing 200-250 grams were used. Rats were housed in an animal house under appropriate conditions of temperature (25 °C), humidity, light and ventilation. All rats were fed standard rat feed and had free access to food and water. Working with animals at all stages was based on the research laboratory instructions of the Islamic Azad University of Falavarjan, Isfahan.

Development of experimental diabetes
Streptozotocin (STZ) (Sigma, America) was used to induce diabetes in rats as a single intraperitoneal dose (60 mg/kg bw) dissolved in cold saline solution. Three days after the injection, fasting blood glucose level was measured by a glucometer (brand???, Country???) with a drop of tail blood to confirm diabetes. Rats whose blood glucose concentration was higher than 250 Mg/dL were considered diabetic and entered the next stage for treatment. The body weight of rats was measured before the beginning of the treatment and during four weeks. Rats were randomly divided into four groups of six and each group was kept in a separate cage. The first group (control group or non-diabetic negative control group) was treated with normal saline. The second group (positive diabetic control) received STZ, but they did not receive the extract. The third and fourth groups were diabetic rats daily treated with intraperitoneal administration of methanol extract of Vaccinium macrocarpon at a dose of 150 and 250 mg/kg bw. Treatment began three days after rats became diabetic and continued up to thirty days. Then, the rats were anesthetized by ketamine and blood was collected from their heart for biochemical assessment of the serum.

Measurement of biochemical parameters
HDL and cholesterol were measured by photometric method (Pars Azmoon Kit). The rats’ weight was determined at the beginning and end of the study. The serum glucose levels were measured at the end of each week. To measure serum protein carbonyl, first, two 2-ml Eppendorfs (sample and blank) were separately used. Serum samples were diluted with sterile saline at a ratio of 4:1 and added to the contents of Eppendorfs, DNPH (500 µl) dissolved in 2N HCL was added, as well. Only 2N HCL (500 µl) was added to the blank Eppendorf. Eppendorfs were placed in the dark for an hour and then ice-cold
TCA (20%) was added to both Eppendorfs to precipitate the proteins. Then the contents of both Eppendorfs were centrifuged for three minutes at 8-10 °C at 11000 g and the supernatant was discarded and combined ethanol/ethyl acetate was added to the plate formed in each Eppendorf in three phases and they were centrifuged for 5 minutes at 8 to 10° C at 8000 g. The superficial liquid after the centrifuge process was discarded. In the end, 6 m guanidine hydrochloride was added in three phases to the plate and incubated at 37 °C. Then the optical absorption of samples was measured at a wavelength of 370 nm and protein carbonyl concentrations were calculated (30).

Malondialdehyde was analyzed as a lipid marker to assess the lipid peroxidation. Under acidic conditions and at 95 °C, one molecule of malondialdehyde reacts with two molecules of thiobarbituric acid forming a pink complex. First, the serum proteins were precipitated with trichloroacetic acid and after it was separated by centrifugation, the solution was used to measure malondialdehyde with optical absorption spectrophotometry colored complex at a wavelength of 535 nm (31).

The activity of organophosphate hydrolase or paraoxonase (PON-1) was determined by calculating the initial speed of the paraoxon substrate hydrolysis to paranitrophenol. To this end, based on Beltowski protocol, a solution containing 100 mM tris-hydrochloric acid and 2 mM calcium chloride at a final concentration of 2 mM paraoxon was prepared at a pH of 8 (measurement mix). Finally, by adding serum (20 µL) containing enzyme to the mixture in the cuvette (steady state), formation of P-nitro phenol was monitored by a spectrophotometer at a wavelength of 412 nm. All experiments were performed twice. In the end, serum paraoxonase (PON-1) activity was calculated by the following formula per µmol/cm (32).

\[ A/T.F = \frac{\text{Paraoxonase activity}}{\text{Paraoxonase activity}} \]

\[ F = \frac{\text{VT/VS}}{\varepsilon 412} \]

\[ \text{VT} = \text{total volume (µL)} \]

\[ \text{VS} = \text{sample size (µL)} \]

\[ A = \text{change in absorbance} \]

\[ T = \text{Time (min)} \]

\[ \varepsilon 412 = \text{molar extinction coefficient of paraoxonase per µmol/cm (0.01829)} \]

One-way analysis of variance (ANOVA) test was used to evaluate the significant difference between the means and repeated measures was used to compare the mean weight at the beginning and end of the study, and the mean serum glucose of rats in four consecutive weeks in SPSS-21. Differences were considered statistically significant if P<0.05.

**Results**

The results showed that administration of Vaccinium macrocarpon methanol extract to diabetic rats has hypoglycemic effect and serum glucose levels in diabetic rats treated with the extract significantly changed compared to the control group (Figure 1).

The results of the administering Vaccinium macrocarpon extract and comparison between the groups in Table 1 show a significant effect on cholesterol, HDL, protein carbonyl, malondialdehyde and serum paraoxonase activity. The results also indicate that the extract has hypolipidemic effect and serum cholesterol in diabetic groups treated with methanol extract 250 mg/kg (high concentration) is significantly different compared to negative control group (non-diabetic control) (P<0.05). The findings suggest that HDL levels in the group treated with high concentrations of Vaccinium macrocarpon extract has a significant difference compared to diabetic positive control group (diabetic control) and the group treated with a concentration of 150 mg/kg (low concentration). Furthermore, there is a significant difference between the group treated with low concentration and control groups and the group treated with high concentration. The diabetic control group has significant
difference compared to experimental groups treated in both concentrations and non-diabetic control group and also the non-diabetic negative control group has significant difference compared to the group treated with low concentration and diabetic control. The comparison between the groups treated with both concentrations and control groups shows a significant difference in terms of protein carbonyl and malondialdehyde and the control groups are significantly different (Table 1). The results of comparing paraoxonase activity between experimental groups show that the group treated with methanol extract has a significant difference with diabetic control group at high concentration, and also the group treated with low concentration of methanol extract is significantly different from the control groups and the control groups themselves are also significantly different from each other (P<0.05) (Table 1).

In this study, the weight of rats was measured at the beginning and end of the study. The mean weight of the rats in four groups changed significantly. Comparison of the mean weight of rats between experimental and control groups showed that treatment with the extract could partially compensate for the weight loss in diabetic rats. The results of the comparison between the groups showed that the group treated with methanol extract at both concentrations and diabetic positive control group were only significantly different from non-diabetic control group. In addition, non-diabetic control group shows a significant difference with all experimental groups (P<0.05) (Figure 2).

**Figure 1: Mean serum glucose in groups for four consecutive weeks**

Results are expressed as mean ± SD value.

a: Statistically significant difference with methanol extract 250
b: Statistically significant difference with methanol extract 150
c: Statistically significant difference with diabetic positive control group
d: Statistically significant difference with non-diabetic negative control group
Table 1: Comparison of biochemical factors in two experimental groups treated with methanol Vaccinium macrocarpon extract and diabetic and non-diabetic control groups (comparison of biochemical parameters in four groups)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment with methanol extract 250 mg/kg</th>
<th>Treatment with methanol extract 150 mg/kg</th>
<th>Diabetic positive control</th>
<th>Non-diabetic negative control</th>
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<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>80.83±4.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>72.00±6.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>110.00±20.27</td>
<td>64.60±1.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein carbonyl (nmol/mg)</td>
<td>44.67±3.67&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>33.17±6.52&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>23.80±4.21&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>49.17±3.76&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malondialdehyde (µmol/dl)</td>
<td>18.88±5.32&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>15.37±3.08&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>32.81±6.67&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>5.54±3.32&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td>Paraoxonase (µu/l)</td>
<td>0.763±0.084&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.83±0.025&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.93±0.078&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>0.23±0.026&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td>HDL (mg/dl)</td>
<td></td>
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<tr>
<td>Protein carbonyl (nmol/mg)</td>
<td></td>
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<td>Malondialdehyde (µmol/dl)</td>
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<td>Paraoxonase (µu/l)</td>
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</table>

Results are expressed as mean ± SD value.
- a: Statistically significant difference with methanol extract 250
- b: Statistically significant difference with methanol extract 150
- c: Statistically significant difference with diabetic positive control group
- d: Statistically significant difference with non-diabetic negative control group

Discussion
The results showed that Vaccinium macrocarpon extract has positive effects on lipid profile abnormalities and improved glucose metabolism disorders in STZ-diabetic rats. The results showed that Vaccinium macrocarpon extract significantly reduced glucose, cholesterol, protein carbonyl and malondialdehyde in diabetic rats treated with the extract compared to diabetic rats without treatment, but significantly increased HDL and paraoxonase activity. Studies by Takikawa et al. (2010) on Vaccinium macrocarpon showed that the plant...
contains a lot of anti-sinusoids. Anti-sinusoids are bioflavonoid complexes in Vaccinium macrocarpon with antioxidant, anti-cancer and anti-inflammatory properties. The results on rats with type II diabetes suggest that a diet containing Vaccinium macrocarpon extract improves hypoglycemia through the activation of protein kinase activating AMP (AMPK) (33). In human studies by Abdo et al. (2006) and Watson (1928) the anti-diabetic effect of Vaccinium macrocarpon was confirmed (18,34). Vaccinium macrocarpon, rich in antioxidants, can prevent increased lipid oxidation after induction of diabetes and flavonoids in this plant increase antioxidant activity and decrease lipid peroxidation, which justifies decreased levels of malondialdehyde in groups treated with the extract compared to diabetic positive control group. Reduced concentration of malondialdehyde as an indicator of oxidative stress in response to Vaccinium arctostaphylous can be attributed to polyphenolic compounds of this plant and its strong antioxidant activity. The findings show that this plant can be considered as a treatment for preventing atherosclerosis in high-risk patients such as hyperlipidemic patients. Inhibition of LDL oxidation by preventing oxidative stress is useful in preventing the development of atherosclerosis which is consistent with the results of the present study (35). In the study of Lee et al. (2008), daily administration of Vaccinium macrocarpon for patients with type II diabetes reduced LDL and total cholesterol and increased HDL levels, which is consistent with the results of the present study (29). Hyperglycosemia in diabetes can increase glycation and oxidative damage in intracellular proteins and plasma leading to the production of nitrotyrosine and protein carbonyl compounds as an early and stable marker for protein oxidation. Increased levels of protein carbonyl in streptozotocin-diabetic rats is reported in different studies, which is consistent with the results of the present study in the diabetic positive control group without treatment with Vaccinium macrocarpon extract (36). Studies show that the inhibition of oxidative processes in diabetic patients can reduce the incidence and progress of late complications in these patients; therefore, supplementation with dietary bioactive compounds such as antioxidant phytochemicals can be a good strategy for reducing oxidative stress and its complications. In addition, consuming substances with antioxidant properties for inhibiting PC is a new challenge for the treatment of diseases associated with carbonyl stress such as diabetes and metabolic syndrome (37). Bigdeli et al. (2012) showed that consuming flavonoid quercetin and aerobic exercise significantly increased antioxidant enzymes in STZ-diabetic rats. In addition, exercise reduced PC in diabetic rats, while the changes were higher in diabetic rats treated with flavonoid quercetin along with aerobic exercise (38). Given that Vaccinium macrocarpon has relatively strong antioxidant properties (39) and its fruit is rich in quercetin and isoquercetin (40), the reduction in protein carbonyl in groups treated with Vaccinium macrocarpon extract can be attributed to strong antioxidant properties and flavonoid quercetin in this plant. Conditions such as diabetes, thyroid diseases, metabolic syndrome, renal failure and aging are associated with reduced activity of paraoxonases (41,42). Quercetin is a flavonoid whose positive effect on PON-1 activity was reported in vivo and in vitro (43). Various studies were conducted on the effect of different fruits that are a mixture of flavonoids and other compounds on the activity of this enzyme. For example, administration of pomegranate juice for rats with apo E deficiency increased paraoxonase activity 43% (44). Flavonoids that have high antioxidant activity can increase paraoxonase activity and strongly prevent atherosclerosis. Few studies have investigated the effect of antioxidant
The effect of the methanol extract of Vaccinium macrocarpon on PON-1 activity (45). It seems that flavonoids compounds in Vaccinium macrocarpon could increase serum paraoxonase activity as an antioxidant enzyme which is consistent with the studies of Aviram and Boich Saadatmandi. Polysaccharides, flavonoids, glycoproteins and polypeptides, steroids, alkaloids and pectin in medicinal plants can well justify hypoglycemic and hypolipidemic properties of some plants used to treat diabetes, such as Vaccinium macrocarpon in this study in terms of preventing biochemical changes in blood.

Conclusion
The results showed that Vaccinium macrocarpon extract has a positive effect on the improvement of impaired lipid profile, increased plasma antioxidant capacity and improved glucose metabolism disorder in streptozotocin-diabetic rats. Vaccinium macrocarpon extract contributes to control of diabetes and lipid disorders and is able to reduce oxidative stress through reduced malondialdehyde and protein carbonyl and activation of paraoxonase. Given that these medicinal plants have fewer side effects than chemical compounds; these plants can be used as valuable alternatives to chemical drugs by further research on the clinical effect of the extracts.

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Conflict of interests
The Authors declare that there is no conflict of interest regarding the authorship or publication of this paper.

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