

## Bioactive components and the effect of hydroalcoholic extract of *Vaccinium myrtillus* on postprandial atherosclerosis risk factors in rabbits

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### ABSTRACT

**Background and Objective:** The important contribution of postprandial state to cardiovascular disease is increasingly being recognized. Diet rich in antioxidant may have beneficial effects on preventing coronary heart disease. This study was therefore aimed to evaluate bioactive components, antioxidant activity and postprandial atheroprotective effects of *Vaccinium myrtillus* (VM).

**Methodology:** Male rabbits weighing  $2.15 \pm 13$  gr were randomly designed into 3 groups of 8, using the following regimens: basic diet, hypercholesterolemic diet and hypercholesterolemic diet+VM. The concentration of total cholesterol (TC), LDL cholesterol, HDL cholesterol, Alanin aminotransferase (ALT), aspartate aminotransferase (AST), fibrinogen, factor VII, nitrite and nitrate were determined in rabbits.

**Results:** *Vaccinium myrtillus* decreased LDL-c, TC and ApoB, liver enzymes (ALT, AST) and inflammatory factors, while endothelial markers (nitrate and nitrate) had subtle decrease in VM treated rabbits.

**Conclusions:** The results of this study suggest that consumption of VM, rich in antioxidant may have beneficial effects on preventing coronary heart disease and atherosclerosis by decreasing remnant lipoprotein values in postprandial state.

**KEY WORDS:** *Vaccinium myrtillus*, Atherosclerosis, Cholesterol, Coronary heart disease.

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### INTRODUCTION

Dietary fats and oils are well known to affect serum cholesterol homeostasis and play an important role in the development of atherosclerosis<sup>1-3</sup> heart disease and cancer which are the main causes of death.<sup>4,5</sup> The risk of coronary heart disease (CHD) is progressively increased with higher values for serum total cholesterol.<sup>6</sup> It has been suggested that coronary artery disease was due to the postprandial accumulation of triglyceride-rich lipoprotein remnants,<sup>7</sup> and the association between metabolic abnormalities and cardiovascular disease has been studied largely during fasting conditions.<sup>8-10</sup> However, the important contribution of postprandial state to cardiovascular disease is increasingly being recognized.<sup>11</sup> Most individuals

spend the majority of non-sleeping hours in the postprandial state. Importantly postprandial hyperlipidemia may in fact be a better predictor of cardiovascular events than fasting lipids.

The adverse effect of postprandial triglycerides is thought to be mediated by pro-atherogenic lipolysis products of lipoproteins, such as remnant lipoproteins and fatty acids, and even a transient increase in these factors may worsen vascular function.<sup>12-14</sup> The association between non-fasting triglyceride (TG) concentrations and risk of coronary death was assessed in 37546 Norwegian male participants, aged 35-49 years, without a history of cardiovascular disease (CVD) and diabetes.<sup>13,15</sup> This analysis detected a statistically significant association between non-fasting TG and coronary death during an average of 9 years of follow-up.<sup>16</sup> In a large cohort study, non-fasting TG showed a 5-fold risk of death due to coronary heart disease in women with a non-fasting TG concentration of 3.5 mmol/l or more compared to those with a level of less than 1.5 mmol, even after adjustment for traditional coronary risk factors.<sup>13</sup> A prospective study conducted in 14916 men aged 40 to 84 years, non-fasting TG concentrations significantly predicted future risk of myocardial infarction.<sup>17</sup> Based on the time after the last meal for baseline blood draws, 26509 initially healthy women in Women's Health Study<sup>18</sup> were stratified into fasting (8 and more hours since last meal) and non-fasting groups (meal within 8 hours prior to blood collection).

After adjustment for standard cardiovascular risk factors including total and HDL cholesterol, diabetes status, body mass index (BMI) and C-reactive protein (CRP), fasting TG levels were not associated with incident of cardiovascular events over an 11-year follow-up period. On the other hand, non-fasting triglycerides maintained a strong relationship with future cardiovascular events even in the fully adjusted analyses. Moreover, after stratification by time since the last meal, TG concentrations measured 2-4 hours after the last meal were the strongest predictor of CVD events. It has been shown that diet low in saturated fat and rich in antioxidant may have beneficial effects on preventing coronary heart disease.<sup>19</sup> From many years ago, plants have been used for medical purposes. Nowadays, it is estimated that approximately 80% of the world populations benefit from herbal medicine. Herbs and spices are generally considered safe and proved to be effective against certain ailments.<sup>20,21</sup>

Recent investigations both in humans and animals showed beneficial effects of herbs such as Soy protein, Grape seed oil, cornelian cherry and apple juice on serum lipid profile.<sup>21-25</sup> *Vaccinium myrtillus* L.

contains a range of micronutrients which are essential for health. It contains a high level of vitamin C (ascorbic acid), folic acid, resveratrol and piceatannol.<sup>26</sup> It may have additional health benefits because it is also rich in phytochemicals such as anthocyanins and flavonols which have antioxidant activities.<sup>27</sup> The anthocyanins of *Vaccinium myrtillus* also exhibit a range of biologic effects, including antioxidant,<sup>28</sup> anti-inflammatory<sup>29</sup> and vasodilatory actions.<sup>30</sup> High levels of phenolics-mainly anthocyanins<sup>31</sup> are considered to be the pharmacologically active and health-promoting, due to their anti-oxidative properties. Oxidative stress plays an important role in the initiation and progression of many heart diseases. Due to antioxidant and free radical scavenging activities of *Vaccinium myrtillus*, we planned to study the postprandial atheroprotective effect of hydroalcoholic extract of *Vaccinium myrtillus*, by measuring lipid profiles, blood glucose level and the factors effective on the function of blood vessels (nitrite and nitrate). Anthocyanin, flavonoid and phenolic contents and antioxidant activity of the extract was also evaluated.

## METHODOLOGY

Fresh *Vaccinium myrtillus* was purchased from the local market in June and authenticated by a botanist in Medical Plants Research Center of Shahrekord University of Medical Sciences. A voucher specimen was deposited in its herbarium (Number 264). *Vaccinium myrtillus* was air dried and milled into fine powder. Then, it was extracted in cold maceration with ethanol 80% for 48 hours. To standardize the powder of plant, some factors like anthocyanins and polyphenoles were measured as follows:

**Measurement of anthocyanin content in *Vaccinium myrtillus*:** Anthocyanin assay was performed using this method.<sup>32</sup> with a slight modification. The Aloe vera powder extract (1g) was added to 100 ml ethanol 95% and 1.5 ml normal hydrochloric acid and kept in refrigerator at 2-4 centigrade degrees for 12 hours. Then, the mixture was filtered and the residue was repeatedly washed with acidic ethanol solvent, and finally the volume of the solution was brought to 250 ml. Then, its absorption was determined in a one-centimeter cell in the wavelength of 535 nm and its anthocyanin content was measured by the following formula:

$A/98.2 \times 25000 = \text{The total anthocyanin level (mg per 100 grams of sample).}$

A = Absorbance sample

**Flavonoid compounds determination of the extract:**

Aluminum chloride colorimetry and Rutin method was used to assay the total flavonoids.<sup>33</sup> First, standard solutions (Rutin in methanol 60%) with concentration levels of 25, 50, 100, 250 and 500 ppm were prepared. Then 1 ml from these solutions was transferred into test tubes and mixed with 1 ml of chloride aluminum 2%. Then, 6 ml potassium acetate 5% was added and the optical density level was read after 40 minutes at 415 nm wavelength. The concentration levels of the standard solutions were assayed in three intervals. In order to measure the overall level of flavonoids in the extracts, 0.01-0.02 g of the extracts was dissolved in methanol 60%, reaching 10 ml. Then, using chloride aluminum colorimetry the total level of flavonoids was measured. However, instead of using the standard solution, 1 ml of the extract was added. The total flavonoid level was calculated in mg per one gram extract, equivalent to Rutin.

**Total phenolic compounds determination of the extract:**

Total phenolic compounds were assayed equivalent to Gallic acid using Folin-Ciocalteu colorimetry.<sup>34</sup> The standard solutions were prepared with concentrations of 12.5, 25, 50, 62.5, 100 and 125 ppm of gallic acid in methanol 60%. Then, 0.1 ml from each sample was transferred into a test tube and 0.5 ml Folin-Ciocalteu 10% was added as reactive agent. The solutions were left for 8 minutes at room temperature and then 0.4 ml sodium carbonate 7.5% was added. The tubes were maintained for 30 minutes at the laboratory temperature and then assayed in three intervals by a spectrophotometer (UnicouV 2010) at 765 nm wavelength. To measure the overall phenol in the extracts, 0.01-0.02 g of the extracts was solved in 60% methanol, reaching 10 ml and then, using Folin-Ciocalteu method, the overall level of phenol was measured. However, instead of using the standard solution, 0.1 ml extract solution was added. Finally, the overall phenol level was obtained from the read optical density in mg/g extract in Gallic acid equivalent.

**Antioxidant activity determination of the extract:**

b-carotene model was employed to measure the antioxidant activity of the extract.<sup>35</sup> 0.5 ml chloroform, 5 ml b-carotene (0.2 mg), 20 ml linoleic acid (20 mg) and 0.2 ml Tween 40 were mixed in a suitable container and incubated at 50°C for 10 minutes in order to remove the solvent. The solution was diluted with distilled water and mixed with 4 ml of aliquots in the following manner. The control solution was prepared including 0.2 ml ethanol and 0.2 ml of the extract sample with 0.15 ml ethanol

and 0.05 ml turmeric extract. The optical density of the control group was recorded at t=0 and t=90 at 470 nm wavelength and similar to the standard group. The samples were incubated in a bain-marie at 50°C. The antioxidant activity was measured on the basis of the ability of the samples in preventing the washing of b-carotene. The antioxidant activity was calculated through Formula 1 below.

$$(1) AA = 100 [1 - (A_o - A_t) / (A_o - A_o^o)]$$

Where,

$A_o$ : the optical density at t = 0

$A_t$ : optical density of the sample at t = 90

$A_o^o$  and  $A_o^o$ : as optical density values in the control samples at t=0 and t=90, respectively.

**Animals:** 24 male rabbits weighing  $2.15 \pm 13$  gr were obtained from the Razi Institute (Karaj, Iran). The rabbits were housed in clean polypropylene cages keeping 4 rabbits per cage and kept for two weeks under the controlled temperature ( $22 \pm 2^\circ\text{C}$ ) with a 12 hour light and 12 hour dark cycle. The animals were fed with basic diet for two weeks in the experimental environment before the experiment was conducted. All experiments were conducted according to ethical standards and protocols approved by the Ethical Committee of Shahrekord University of Medical Sciences, Iran.

The animals were evenly divided into three groups of eight rabbits in each group and treated as follows: Group 1: Normal control group (NC), in which rabbits were fed with basic diet.

Group 2: High cholesterol group, in which the animals received 1% hypercholesterolic diet.

Group 3: High cholesterol + *Vaccinium myrtillus* group, in which the animals received 1% hypercholesterolemic diet plus 2% *Vaccinium myrtillus*.

At the beginning of the experiment (after 15 hours fasting) and after 3 hours of treatment the blood samples of each rabbit were collected and immediately stored in 2 different test tubes (containing 0.5cc citrate) and serum were separated by 20 min centrifugation at 3500 rpm.

Blood glucose level was measured using Biosystem kits and the amount of HDL Cholesterol, LDL-cholesterol, triglyceride, total cholesterol, alanin amino transferase (ALT) and aspartate amino transferase levels were determined using pars-azmoon commercial kits (Iran) on the Hitachi 902 Autoanalyzer (Japan). For fibrinogen measurement, blood was collected and stored in citrate tubes and the plasma was measured by coagulation method using Mahsayaran kit (Iran).

**Statistical Analysis:** All the statistical analyses were carried out by SPSS software. The data were

represented as mean $\pm$ SD. One-way Analysis of Variance and Dunnett's multiple comparison tests were used to assess the differences. P Value <0.05 was considered as statistically significant.

## RESULTS

After analyzing each 100 g of *Cornus mas* L. powder yielded  $11 \pm 0.4$  g ethanolic extract. The calculated levels for flavonoid and phenolic compounds were  $17.98 \pm 1.9$  mg/g Rutin equivalent and  $215.56 \pm 2.88$  mg/g Gallic acid equivalent, respectively. The amount of anthocyanins in *Vaccinium myrtillus* L. was 398 mg/100 g and its antioxidant activity was 46%. In the high cholesterol treated group, glucose, triglyceride (TG), fibrinogen, ALT, AST, total cholesterol (TC), LDL-C levels increased in comparison with control group ( $P < 0.05$ ). Adding *Vaccinium myrtillus* extract to high cholesterol treated rabbits decreased LDL-C, cholesterol and ApoB ( $P < 0.05$ ), but not glucose and triglyceride levels (Table-I).

Fibrinogen decreased after *Vaccinium myrtillus* treatment but factor VII changes were not significant between three groups of rabbits. *Vaccinium myrtillus* decreased liver enzymes (ALT & AST) and inflammatory factors. The endothelial markers (Nitrite and nitrate) had subtle decrease ( $P > 0.05$ ) in *Vaccinium myrtillus* treated groups.

## DISCUSSION

Coronary vascular disease is increasingly being recognized the important contribution of postprandial state.<sup>36</sup> Significant evidence indicates that oxidative stress leads to cell and tissue injury.<sup>37-39</sup> Antioxidants represent a first line of defense against free radicals and reactive oxygen species (ROS) that target lipids, proteins and nucleic acids.<sup>40</sup> A very promising way to overcome this is to use vegetables and fruits with antioxidant properties.<sup>41</sup> The presence of antioxidants in many herbs and species can have beneficial protecting effect on retarding the process of lipid peroxidation and atherogenesis. Recently herbal medicines have gained the interest of many researches.<sup>42</sup> The extract of *Vaccinium myrtillus* showed considerable antioxidant activity which is in accordance with the results of previous studies.<sup>43</sup> Glucose, TG, fibrinogen, ALT, AST, TC, LDL-C levels increased in high cholesterol treated rabbits (Group2) in comparison with normal control rabbits (Group1) which showed the model was successful in inducing hyperlipidemia.

In our study, there was no significant changes regarding serum glucose level in *Vaccinium myrtillus* treated group while in Roghani et al study, *Vaccin-*

Table-I: Mean ( $\pm$ SD) of biochemical factors after treatment with *Vaccinium Myrthillus* LDL-C (low density lipoprotein), TG (triglyceride), TC (total cholesterol), ApoB (apolipoprotein B), Alanine aminotransferase (ALT), aminotransferase Aspartate (AST).

Biochemical Factors	Groups		
	Normal Diet	High Cholesterol diet	<i>Vaccinium Myrthillus</i>
LDL-C (mg/dl)	24.13 $\pm$ 1.26*	39.31 $\pm$ 3.20	19.38 $\pm$ 1.9*
fibrinogen (mg/dl)	218.9 $\pm$ 2.6*	251 $\pm$ 4.6	213.9 $\pm$ 5.1*
TG (mg/dl)	50.50 $\pm$ 1.43*	120.56 $\pm$ 5.45	115.88 $\pm$ 14.52
TC (mg/dl)	56.63 $\pm$ 0.68	91 $\pm$ 3.37	70.25 $\pm$ 2.09*
ALT (u/l)	26.63 $\pm$ 0.50*	40 $\pm$ 1.34	29.13 $\pm$ 0.64*
AST (u/l)	29.75 $\pm$ 0.53*	43.22 $\pm$ 2.63	35.63 $\pm$ 1.43*
Glucose (mg/dl)	51.25 $\pm$ 3.12*	132 $\pm$ 3.57	143.75 $\pm$ 5.87
Factor VII (%phy. activite)	295.7 $\pm$ 2.5	298.1 $\pm$ 5.7	296 $\pm$ 5.5
ApoB (mg/dl)	27.88 $\pm$ 0.88	30.78 $\pm$ 1.02	27.13 $\pm$ 0.44*
Nitrate ( $\mu$ Mol/l)	305.6 $\pm$ 108.8	430 $\pm$ 36.7	170.8 $\pm$ 97.3
Nitrite ( $\mu$ Mol/l)	250.4 $\pm$ 10	249.3 $\pm$ 10.4	211.2 $\pm$ 45.6

\* $p < 0.05$ : significant differences between high cholesterol group and others.

*ium myrtillus* treatment of diabetic rats did have a significant hypoglycemic effect after 2 and 4 weeks of the experiment ( $P < 0.001$ ).<sup>43</sup> This may suggest that *Vaccinium myrtillus* has a significant hypoglycemic effect in diabetic patients and can decrease high level of glucose but significant changes not observed regarding serum glucose level in our study. This may be because of different model of study and the lower serum glucose level in rabbits in comparison with diabetic rats.<sup>44</sup> Remnant lipoproteins, as well as the oxidized low density lipoprotein (Ox-LDL), is easily taken into the macrophage in the arterial wall, promoting foam cell formation of macrophages and forming atherosclerotic lesion.<sup>45</sup> In the past few decades, numerous clinical trials have established the efficacy of lowering low-density lipoprotein cholesterol (LDL-C) for the prevention of coronary heart disease.<sup>46</sup> Postprandially, LDL-C and cholesterol levels significantly decreased in rabbits treated with *Vaccinium myrthillus* in comparison with high cholesterol treated rabbits which suggest that *Vaccinium myrtillus* may be useful to prevent coronary heart disease. *Vaccinium myrtillus* reduced TG levels of rats on hyperlipidaemic diet but the reduction was not significant while indicated that active constituent(s) of *Vaccinium myrtillus* L. leaves may be potentially useful for treatment of dyslipidaemia.<sup>47</sup>

Several carbohydrates are able to regulate lipemia and triglyceridemia. In Kazumi et al study, feeding glucose in Wistar fatty rats produced a similar increase in the activity of NADPH-generating enzymes and triglyceride concentrations or hepatic fatty acid synthetase activity which suggests that glucose stimulates triglyceride production.<sup>47</sup> Vaccinium myrtillus treatment in our study did not significantly decrease glucose which can justify not significant reduction in triglyceride level. Liver enzymes (AST, ALT) were significantly decreased in Vaccinium myrtillus treated rabbits. This confirm the protective effect of aqueous extract of Vaccinium myrtillus against oxidative stress toxicity in isolated rat hepatocyte,<sup>48</sup> suggesting that antioxidants components of Vaccinium myrtillus extract may prove potentially useful effects against the intracellular oxidative stress. In Naderi et al study, total extract of black pepper decreased AST release from hepatocytes which was attributed to antioxidant components of black pepper.<sup>49</sup> Vaccinium myrtillus is rich in such antioxidant that can justify the result of our study.

The development of atherosclerosis is a dynamic process that results from excessive inflammatory and fibroproliferative responses.<sup>50</sup> NO may normally prevent atherosclerosis by inhibiting smooth muscle cell proliferation, inhibiting platelet aggregation and adhesion, and inhibiting leukocyte activation and adhesion.<sup>51</sup> In our study plasma levels of nitrites/nitrates (as endothelial markers) decreased after Vaccinium myrtillus treatment, but this was not significant. Multiple administrations of Vaccinium myrtillus may result in a significant decrease in serum nitrites/nitrates levels. Fibrinogen is an acute phase protein and its level increases in many acute diseases.<sup>52</sup> Because fibrinogen is one of the risk factors of cardiovascular disease,<sup>53</sup> its level was measured in this study. Hypercholesterolemic rabbits had higher baseline level of fibrinogen compared to normal control group and administration of Vaccinium myrtillus prevented this increase. Result of 11-year follow up suggested that factor VII activity is an independent predictor of cardiovascular mortality of a Sicilian population.<sup>54</sup>

In our study, factor VII change was not significant between 3 groups of rabbits. This may be because of short duration of the study because its production in liver takes relatively a time. It is recognized that atherosclerotic risk of postprandial hyperlipidemia is derived from an increase of remnant lipoproteins. Increase of remnant lipoprotein values in

postprandial state is one of coronary risk factors.<sup>55-58</sup> This result suggests that reduction in lipoprotein values may have protective effect against postprandial hyperlipidemia atherosclerosis. Vaccinium myrtillus effectively reduced LDL and total cholesterol which suggest that consumption of Vaccinium myrtillus, rich in antioxidants, may have beneficial effects on preventing coronary heart disease and atherosclerosis. The present findings indicate that consumption of Vaccinium myrtillus is potentially useful for treatment of hyperlipidemia in rabbits. Furthermore, it makes further studies crucial to understand the dose response of this herb.

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