Antioxidant Effect of Bilberry on Oxidative Stress Caused by Acute Exercise in Rats

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\textbf{Abstract}

This study, it was aimed to determine the antioxidant effect of bilberry extract in decreasing the oxidative stress caused by acute exhaustive exercises in rats' blood and liver tissues. 27 Spraque-Dawley type male rats were divided into four groups (control, exercise, bilberry and bilberry+exercise groups). Bilberry extract was administered using gavage once daily. Before blood and liver tissues were taken, the rats ran in a treadmill at a speed of 25 m/min (1.5 km/h) at a slope of 0 for about 1 hour or until exhaustion. Compared to the control group, serum GSH levels and GPx activities did not have a significant change in the bilberry, acute exercise and bilberry + acute exercise groups; while serum MDA levels decreased significantly. When compared to the control group, liver GPx activity significantly increased in the bilberry and bilberry+acute exercise groups. Again, it was determined that liver GSH level significantly increased in bilberry+acute exercise group. The results of this study demonstrate that bilberry extract may provide antioxidant protection against a potential oxidative damage as it causes an increase in hepatic GPx activity and GSH levels in rats exposed to acute exhaustive exercise.

\textbf{Keywords:} Antioxidants, acute exhaustive exercise, bilberry, glutathione, glutathione peroxidase, malondialdehyde

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1. Introduction

While exercise has many beneficial effects on health\textsuperscript{1}, there are many findings that a certain amount of oxidative damage occurs in muscle, liver, blood, and other tissues during exercise, and reactive oxygen species (ROS) and free radical production increase especially in intense exercises\textsuperscript{2-4}.

The most significant biological change that occurs during exercise is the increase in the rate of oxygen consumption.\textsuperscript{5} In parallel with the increase in oxygen consumption, the production of the free radicals is also accelerated.\textsuperscript{6, 7} Excessive ROS production can seriously block the antioxidant defense, causing cellular homeostasis to change. Thus, lipids can initiate oxidative stress, which causes different cellular damages affecting proteins and nucleic acids.\textsuperscript{8-10} Antioxidant enzymes that are effective at the cellular level against ROS, which is produced during exercise, include SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase) and GSH (Glutathione)\textsuperscript{11}. It has been stated that acute exhaustive exercise may adversely affect the activities of these antioxidant enzymes\textsuperscript{12}. The levels of oxidant and antioxidant molecules that are formed during the exercise vary according to the intensity and duration of the exercise\textsuperscript{2}. Acute exercise leads to muscle tissue damage, lipid peroxidation in membranes and the formation of free radicals\textsuperscript{4}. Regular and short-term submaximal exercises activate antioxidant systems more often while the damaging oxidant system becomes more active in intense and long exercises\textsuperscript{6-13}.

Advances in scientific technology have allowed us to understand the relationship between diets and diseases, in addition, the protection of our health and the biological regulatory roles of the antioxidant nutrients in our bodies have been attracting more interest\textsuperscript{7}. As the wrong nutritional habits and the medical problems emerge, it becomes clear that one of the most basic rules of living healthy and fighting diseases is a healthy nutrition\textsuperscript{14}. In this context, the formation of free radicals and the determination of antioxidant capacity are important in terms of the application of antioxidant diets and/or the use of medicines in order to reduce the risk of catching such diseases\textsuperscript{15}.

It has been shown that various nutrients and nutritional elements have positive effects on our health and that they contribute to the prevention and treatment of certain chronic diseases.\textsuperscript{16} It has recently been reported that bilberry extracts have cellular protective and antioxidant effects against oxidative damage formed through various in vitro models\textsuperscript{17, 18}. These characteristics show that it is a good choice for studies on nutrition\textsuperscript{19, 20}. Studies have also reported that the consumption of this fruit slows down physiological and functional disorders associated with age\textsuperscript{21}. Studies have indicated that this plant has been used in the traditional treatment of liver
diseases, as well as using its characteristics of controlling blood pressure, serum glucose and serum lipid levels, and also urinary antiseptic, anti-inflammatory, antidiabetic and anticancer effects together with its antioxidant properties.

In this study, we aimed to investigate whether there's a protective effect of bilberry, which has strong antioxidant properties against oxidative stress caused by acute exhaustive exercise, on the liver and serum tissues of rats. For this purpose, MDA and GSH levels and GPx activities were measured in liver homogenate and serum tissue specimens and compared with the control group.

2. Material and Methods
2.1 Determination of Experimental Animals and Exercise Protocol
In this study, 27 two months old, male, Spraque-Dawley type rats with average weights ranging from 280-300 g were used. During the experiment, the rats, which were kept in wire cages in the 12/12 hour light/dark cycle of light, at a temperature of 22°C, and in 50-60% humidity, were fed with standard pellet feed and tap water.

The exercising group's running exercises were carried out on a four-way treadmill (May Time 0804 Animal Treadmill) with an electric motor drive. Before starting the exercise protocol, the rats were allowed to run on the treadmill for 10 minutes a day for 5 days at a rate of 10 m/min (0.9 km/h) at a slope of 0 for being familiarised. During acute exhaustive exercise, the rats ran on the treadmill at a speed of 25 m/min (1.5 km/h) at a slope of 0 for about 1 hour or until being exhausted.

2.2 Preparation of Bilberry Extract
The Bilberry fruit collected from Artvin region was dried at 40°C before the analysis and the dried fruit was grounded. For the analyses, about 400 g of grounded dry sample was taken and enough water was added to exceed the sample. The prepared sample was stirred for one day, and it was filtered and diluted with water to a concentration of 100 mg/kg. Antioxidant activities were then monitored.

2.3 Creation of Experiment Groups
Control Group (C, n=6): No extra treatment was applied to rats, which were kept in standard conditions.

Bilberry Group (B, n=7): Bilberry extract was administered to the rats housed under standard conditions at a dose of 1x100 mg/kg/day, using 2 cc gavage daily for 30 days.

Exercise Group (E, n=6): The rats in this group were familiarised with the treadmill for 10 minutes at a speed of 10 m/min (0.9 km/h) at the inclination of 0 for 5 days before starting the exercise protocol. Then, before the tissues were taken, they ran in the treadmill for about 1 hour or until exhaustion at a speed of 25 m/min (1.5 km/h) at an inclination of 0 within the acute exhaustive exercise program.

Bilberry + Exercise Group (B+E, n=8): The rats housed under standard conditions were given bilberry extracts at a dose of 1x100 mg/kg/day, using 2 cc gavage daily for 30 days. They were then acclimated to the treadmill for 10 minutes at a speed of 10 m/min (0.9 km/h) at 0 inclination for 5 days prior to beginning the exercise protocol. On the last day, the 31st day of the experiment, the acute exhaustive exercise was carried out making the rats run on the treadmill for about 1 hour at a speed of 25 m/min (1.5 km/h) at 0 inclination or until exhaustion.

Bilberry extracts were not given to the rats on the day of the collection of the tissues and blood samples were taken intracardially under general anesthesia (when there was no response to the painful stimulus). The liver tissues were taken after the hemorrhagic shock.

2.4 Preparation of the Samples
Blood specimens were placed in tubes without anticoagulants, were kept at room temperature until completely coagulated, and then they were centrifuged at 4°C for 5 min at 3500 x g, and afterward, serum fractions were separated and they were stored at -80°C until biochemical measurements.

2.4.1 Preparation of liver Homogenates
The extracted liver tissue was thoroughly washed with ice-cold isotonic NaCl solution to remove the bloody parts and the wetness was removed with the drying paper. The tissue was stored in a deep freezer at -80°C until the analysis. On the day of the measurements, approximately 300 mg of wet tissue was homogenized in 3 ml of phosphate buffer (50 mM pH 7) for GPx and GSH measurements, and in 1.15% KCl solution for MDA measurement (OMNI International, USA). Tissue homogenates were centrifuged at 10,000 x g for 15 minutes at 4°C, and their supernatants were taken and these supernatants were used for GPx and GSH measurements. Additionally, the MDA measurements were performed using tissue homogenates.

2.4.2 Malondialdehyde (MDA) Measurement
The principle of the experiment is based on the absorbance of the pink colored complex, which is formed by thiobarbituric acid and MDA, and which is spectrophotometrically measured at a wavelength of 532 nm, after the incubation at 95°C. 26 Sodium dodecyl sulphate (SDS) solution (8.1%), 20% acetic acid solution (pH 3.5,
adjusted with NaOH), 0.9% thiobarbituric acid (TBA) solution, n-Butanol/Pyridine (15/1, v/v) solutions were prepared as the measurement reagents. As a standard, 1.1.3.3 tetraethoxypropane (Sigma) was used, and a stock standard solution at 0-200 μmol/L concentration was prepared. Serial dilutions were made from the stock standard to obtain solutions at a concentration range of 0-200 μmol/L, and it was used as the measurement standard.

2.4.3 Glutathione Peroxidase (GPx) Activity Measurement
In the determination of GPx activity, a commercially produced measurement kit (Glutathione Peroxidase Assay Kit, Cat No: 703102, Cayman Chemical, Ann Arbor, MI, USA) was used. GPx activity has been carried out in accordance with the manufacturer's recommendations on the measuring method. Enzyme activity was calculated using the molar absorbance coefficient of NADPH (0.00373 μM⁻¹.cm⁻¹ for 0.6 cm microplate light path). GPx activity was expressed as U/mL (for serum) or U/g protein (for tissue).

2.4.4 Glutathione (GSH) Measurement
The GSH level measurement was performed in accordance with the recommendations of the manufacturer company using a commercial kit (Glutathione Assay Kit, Cat No: 703002, Cayman Chemical, Ann Arbor, MI, USA). GSH level was expressed as mol/L (for serum) or mol/g protein (for tissue).

2.4.5 Protein Measurement
Protein measurement in tissue supernatant samples was performed according to the Bradford method. Protein values were used to calculate the tissue concentration or the specific activity for GSH and GPx.

2.5 Statistical Analysis
Statistical analysis of the data was performed using the PASW Statistics 18.0 (SPSS Inc., Chicago, IL) program. The variables' suitability to the normal distribution was assessed by the Kolmogorov-Smirnov test. Intergroup comparisons were analyzed by One-Way ANOVA LSD Post Hoc test. The differences, where P<0.05, were considered as statistically significant.

3. Results
3.1 Group Weights
When compared with the C group, there were no significant differences in the body weights among the groups. Group weights are shown in Table 1.

| Groups            | Average Rat Body Weights (g) |
|-------------------|-------------------------------|
|                   | First Weighing (1st day) | Final Weighing (30th day) | Difference | % |
| Control           | 6 309 339 +30 +10            |
| Bilberry          | 7 303 318 +15 +5            |
| Exercise          | 6 307 333 +26 +8            |
| Bilberry+ Exercise| 8 305 326 +21 +7            |

3.2 Biochemical Findings
3.2.1 Blood tissue oxidant/antioxidant parameters
Serum GSH, GPx and MDA values measured on the study groups are presented in Fig. 1, while liver homogenate GSH, GPx and MDA values are shown in Fig. 2. Serum MDA, GSH levels and GPx activities of B, E and B+E groups were compared with the C group, there was no significant difference in GSH levels and GPx activities, while serum MDA levels decreased in a significant rate.
C: Control; B: Bilberry; E: Exercise; B+E: Bilberry+Exercise; GSH: Glutathione; GPx: Glutathione peroxidase; MDA: Malondialdehyde

Figure 1: The measured serum GSH, GPx and MDA values, GSH levels and GPx activities (p>0.05 for all of them), *MDA levels of group B, group E, and group B+E (p = 0.011, p = 0.013 and p = 0.0001, respectively)

3.2.2 Liver tissue oxidant/antioxidant parameters

The GPx activity in the liver was significantly increased in the B group and the B+E group when compared to the C group, and the liver GSH level was significantly increased in the B+E group, whereas the MDA concentration did not change significantly (Figure 2).

Figure 2: GSH, GPx and MDA values of the liver tissue. * GPx activities of Group B and Group B+E (p=0.030 and p=0.0001, respectively). ** GSH of Group B+E (p=0.005), *** MDA levels (p=0.711).

4. Discussion

It is known that exercise is a stress source that enhances the oxidative stress by increasing the production of free oxygen radicals, on the other hand it affects the activity of antioxidant enzymes and develops resistance to oxidative stresses\(^2\). Short exercise, which can be evaluated as a system that activates the antioxidant system instead of oxidant system, or sweeps away the oxidant products that have formed, can act as an antioxidant mechanism. It has been shown in many studies that, the duration of exercise and its intensity have effects on this situation\(^28\)\(^-\)\(^31\). It is stated that acute exhaustive exercise enhances the formation of the free radicals and this increase can be proved by the increase of lipid peroxidation, glutathione oxidation and oxidative protein damage\(^32\).

In our study, serum acute MDA levels in groups B, E and B+E decreased significantly after acute exhaustive exercise, when compared to the C group. We suggest that bilberry extract, which has low MDA levels and a high antioxidant power, accelerates the oxidative stress by increasing antioxidant enzyme activities. When the serum antioxidant parameters were examined in our study, it was seen that there was no significant difference in serum GSH levels and GPx activities in groups B, E and B + E when compared to the C group. The
GPx activity was found to be higher in the B and B+E groups than in the other groups, however, this increase was not statistically significant. When studies in the literature are examined, the fact that different results have been observed in GSH and GPx activities only after acute exercise supports our results.

Tavera et al\textsuperscript{33} reported that anthocyanins were observed in the plasma of rats within 30 min, and that plasma antioxidant levels increased significantly after 3-6 hours of feeding compared to the control group, in the group fed with bilberry extract. Rokitkzi et al\textsuperscript{34} observed that there was no significant change in GPx levels in intensely-trained athletes and skiers, while there was an increase in MDA after intense exercise, by antioxidant supplementation 4.5 weeks before long-distance runs in intensely-trained runners and skiers. Sahlin et al\textsuperscript{35} found that MDA and total GSH increased in acute exercise. On the contrary, Thirumalai et al\textsuperscript{36} reported a significant decrease in GPx levels in rats subjected to a 5-day intensive and comprehensive swimming training program. Again, Aguilo et al\textsuperscript{37} reported that there was a significant decrease in serum GPx after acute cycling. The results of these studies have emphasized that acute exhaustive exercises reduce antioxidant capacity and that supplementation of antioxidant agents prior to acute exercise may be beneficial in increasing antioxidant capacity.

When the literature is examined, a large number of studies have been found indicating that short-term or acute intensive exercises increase the oxidative stress.

Alessio et al\textsuperscript{38} reported that MDA did not change in exhaustive aerobic exercise, Duffaux et al\textsuperscript{39} reported that MDA did not show a significant increase after intensive running test in physical education students, and Leaf et al\textsuperscript{40} stated that MDA did not change in before, after and during maximal exercise. Grisham\textsuperscript{41} did not find a significant difference in MDA during acute exercise. Similarly, Dernbach et al\textsuperscript{42} reported that there was no change in plasma MDA levels before and after the 4-weeks of intensive rowing training and during recreation in the athletes. Selamoglu\textsuperscript{43} has found a statistically significant decrease in the MDA in long-distance runners. As in our study, there are many studies that have found a decrease in MDA levels and have supported our results. Celik et al\textsuperscript{44} reported that the football players had a decrease in MDA levels after acute exercise. Aksu et al\textsuperscript{45} reported that acute exercise did not produce oxidative stress in rat brain (prefrontal cortex, striatum and hippocampus) as the result of their study. They explained these findings in the way that the effects of free radicals on different organs may also be different. It has been reported that at least some of these conflicting results regarding MDA levels may be due to changes in the plasma volume that the exercises cause\textsuperscript{46}.

It has been supported by the studies that, although there are numerous known benefits of the moderate level and regular exercises, the acute exhaustive exercise causes oxidative stress and accordingly oxidative damage in the tissues of the many vital organs such as blood, liver, kidney, and brain\textsuperscript{47}. The utilization of antioxidant agents may provide certain protection against the damaging effects of radicals and may also prevent the side effects that may occur by partially inhibiting free radical formation caused by exercise. For example, the use of antioxidants such as C and E vitamins has been reported by the studies to be partially protecting against exercise-mediated oxidative damage in both people and rats\textsuperscript{48}.

Davies et al\textsuperscript{49} reported that free radical concentration, MDA and mitochondrial damage in liver and muscle homogenates of the rats fed with antioxidant diet and having acute exhaustive exercise were only higher than the rats fed with vitamin E, when compared with exercise groups receiving exhaustive exercise but not antioxidant supplementation, while there was a 100\% increase in their levels of MDA. Knez et al\textsuperscript{50} reported a significant increase in MDA levels in both groups in their study on semi-and full-distance triathlon runners, while there was a decrease in GPx levels.

It has been reported that chronic liver disease begins with oxidative stress and that intense stress causes significant DNA damage, structural abnormalities in the liver and hepatocellular carcinoma\textsuperscript{51, 52}. It has been observed that anthocyanins and other phenolic compounds found in berry fruits and plants exhibit antioxidant effects both in damage and inflammation, which occur in the liver via alcohol or other toxins\textsuperscript{33, 34}. In general, the total antioxidant capacity of liver tissue is regulated by GSH, C, E vitamins and possibly by other homeostatic mechanisms including various endogenous components. GSH is a very important antioxidant, which is found in tissues. For this reason, increasing the GSH level may be beneficial in the extinction of the reactive oxygen species in the cell\textsuperscript{42}. Certain studies have shown that deficiency of glutathione in the pathophysiology of many diseases can be prevented or reversed by providing GSH or GSH precursors\textsuperscript{55}. Luo et al\textsuperscript{56} reported that bilberry extract takes part in preventing liver damage and oxidative stress development.

In our study, when the liver homogenate GSH, GPx activity results were compared to the C group. It was observed that there was a significant increase in liver GPx activity of B and B+E groups, there was a significant increase in the liver GSH levels of B+E group compared to the C group, while there was no significant change in MDA concentrations. The increase in liver homogenates of GSH and GPx activity of the Bilberry-consuming groups suggests that it may have originated from bilberry, which is a powerful antioxidant. The studies in the literature are consistent with the values that we have found on the liver homogenates. Talavera et al\textsuperscript{57} found that the amount of liver anthocyanin in rats fed with an anthocyanin-rich diet for 15 days was considerably high. Kalt et al\textsuperscript{58} reported that there were high amounts of anthocyanin in the liver, eye, cortex and cerebellum of pigs fed...
with bilberry for 4 weeks; and Tang et al.\textsuperscript{59} reported that bilberry extract not only reduces liver damage but also decreases the oxidative stress in rats.

Bao et al.\textsuperscript{60} reported that plasma MDA levels were lowest, and the liver GSH and vitamin C levels were the highest in rats fed with 200 mg/kg/day, in their study that they administered the bilberry extract and vitamin C at different doses orally, before their five-day fasting, to the rats, to whom they applied limited hunger stress for 18 hours. Sakakibara et al.\textsuperscript{61}, and Lchiyanagi et al.\textsuperscript{62} reported that the accumulation of anthocyanin is most abundant in the liver, in their studies examining the distribution and absorption of anthocyanins present in bilberry extracts by various organs of mice.

5. Conclusions

As the result, numerous studies have shown that the antioxidant defense system, which is suppressed during the situations that the exercise stress is triggered, can be compensated by the application of antioxidant agents from the outside, and that oxidative stress decreases as a natural end-result of this application. Supplementing antioxidants to those who exercise may be beneficial to reduce oxidative stress and increase antioxidant capacity. Using antioxidant supplements can also increase the performance of those who exercise. For this reason, we believe that further and advanced studies are required.

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