The effect of pomegranate peel hydroalcoholic extract and vitamin E supplementation on strengthening antioxidant defense system in rats under exhaustive exercise stress

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Abstract

Introduction:
Acute exhaustive exercise increases reactive oxygen species, which may decrease antioxidant capacity of the body. Regular exercises and antioxidants consumption can improve antioxidant capacity. The present study aimed to determine the protective effect of pomegranate peel hydroalcoholic extract supplementation on oxidative responses induced by an exhaustive exercise schedule in rats.

Materials and Methods:
Thirty-two male rats were randomly divided into four groups of eight: control group received saline, PPHE200 group received 200 mg/kg pomegranate peel hydroalcoholic extract orally, PPHE250 gorup received 250 mg/kg pomegranate peel hydroalcoholic extract orally, and Vit E group received 5 mg/kg vitamin E orally. After eight weeks of regular exhaustive exercise the activity level of glutathione peroxidase, catalase, reduced glutathione and lipid peroxidation was measured.

Results:
The activity of glutathione peroxidase, catalase and reduced glutathione significantly increased in PPHE200, PPHE250 and vit E groups than in the control (p<0.05). Malondialdehyde level significantly reduced in PPHE200, PPHE250 and vitE groups than in the control (p<0.05).

Conclusion:
The results showed that pomegranate peel hydroalcoholic extract supplementation may be beneficial in improving antioxidant defense system and in reducing oxidative stress induced by an exhaustive exercise.

Keyword: Exhaustive Exercise, Oxidative Stress, Antioxidant

Introduction
Considerable advancements have been achieved regarding medicinal mechanisms of pomegranate and its unique parts in recent decades. It appears that extracts of all parts of pomegranate fruit have therapeutic properties (1). Some reports indicate that the bark, roots, and leaves of pomegranate trees have useful therapeutic properties (2).
Studies have shown that plants rich in anthocyanins, flavonoids, and polyphenols are effective in scavenging free radicals (3, 4). Among the polyphenolic compounds, flavonoids are powerful antioxidants against LDL oxidation. Peroxide and superoxide anions, which collect and remove hydroxyl radicals, are in the pomegranate peel (5, 6) and have a wide range of health effects including positive impact on cardiovascular diseases and generally on the diseases caused by free radicals. These compounds inhibit the membrane lipid peroxidation, chelate metals, and stimulate the activity of enzymatic antioxidants. Pomegranate peel has more antioxidant properties than other parts of the pomegranate because of its polyphenolic compounds (7). The antioxidant activity of polyphenols in pomegranate seeds was investigated by measuring malondialdehyde (MDA) in the brain of rats, and proved that these compounds significantly reduce toxic effects of carbon tetrachloride in rats (8).

Under normal conditions, there is a balance between production and elimination of free radicals in the body. The imbalance between these two processes leads to oxidative stress and multiple pathobiologic changes in the cell macromolecules (9). The production of reactive oxygen and nitrogen species in normal metabolism of the body increases by exposure to ultraviolet radiation and other environmental pollutions in form of oxidants, and disrupts the antioxidant-oxidant balance, and negatively impacts the body cells. MDA, as an important aldehyde from membrane lipid peroxidation, indicates the degree of oxidative stress in the body (10). Enzymatic and non-enzymatic antioxidants are the body's main defense against damage caused by free radicals. The key enzymes of the system are superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR) and catalase (CAT). A major feature of these enzymes is their inducibility under oxidative stress. That is, their amount increases or decreases depending on the type of stress (11).

A review of the literature yielded no study on the effects of hydroalcoholic extract of pomegranate peel on oxidative stress resulting from the intensive physical activity. Furthermore, natural antioxidants neutralize free radicals and have few negative effects compared to synthetic antioxidants. Hence, the present study was conducted to evaluate the effect of hydroalcoholic extract of pomegranate peel and vitamin E on strengthening the antioxidant system of rats under the oxidative stress induced by exhaustive exercise.

Materials and Methods

Animals and exercise protocol

Considering the possibility of losses during gavage, 40 adult male Wistar rats with a mean weight of 220±20 g entered the study, and eventually 8 rats were examined in each group. A week after adaptation, the rats were randomly divided into four groups: control group (CON), PPHE200 group (receiving 200 mg/kg of pomegranate peel hydroalcoholic extract), PPHE250 group (receiving 250 mg/kg of pomegranate peel hydroalcoholic extract), and Vit E group (receiving 5 mg/kg of vitamin E). All groups received a dose of 2 ml by gavage and the control group received the same amount of normal saline. Treatments were applied daily for eight weeks. During the test, animals were moved and treated by the same person and had free access to food and water. Animals were kept in 12-hour light/dark cycle. Animals were placed in a
cylindrical pool with a diameter of 100 cm and depth of 50 cm for exercise. Water temperature ranged 32±2 °C. On the first day of testing, animals swam in the pool for 10 minutes, it increased to 60 minutes within 6 days (12), and this trend continued for eight weeks. In the last exercise session, all groups were placed under exhaustive exercise stress. Exhaustive exercise stress was defined as a lack of animals' movement on the water due to exhaustion that would lead to drowning if continued (13). At this stage, complete care was employed to avoid drowning of the animals. The entire process of working with laboratory animals was according to the international guidelines and the ethics of working with laboratory animals were respected.

**Preparation of pomegranate peel hydroalcoholic extract**

The hydroalcoholic extract of pomegranate peel was prepared by maceration. That is, pomegranate peel was dried at 40 °C in an oven, and the resulting powder was added to hydroalcoholic solution (50% water and 50% ethanol 70%) at a ratio of 1:3. The resulting suspension was stored at 30 °C for 72 hours. The prepared extract was passed through a filter paper, condensed using rotary vacuum distillation, and its solvent was separated.

**Preparation of the serum sample**

After exhaustive exercise, animals were immediately anesthetized using a combination of high levels of ketamine (80 mg/kg) and xylazine (10 mg/kg), and blood samples were taken from the portal vein to the highest possible amount (4-5 ml). Blood samples were stored at room temperature for 30 minutes to separate serum and then centrifuged at 500 g for 15 min at 4 °C. The separated serum was stored at -70 °C for further examination.

**Measurement of lipid peroxidation (MDA)**

MDA was measured using Esterbauer et al. method. That is, 1500 μl of 0.06% TBA and 1000 μl of 1% TCA were added to a tube. Then, 100 μl of the serum sample or homogenized tissue was added. The tubes were placed in a boiling (100 °C) water bath for 30 minutes, and centrifuged for 15 minutes at 1000 RPM after cooling. The absorption of the supernatant was determined using a spectrophotometer at 535 nm against a reagent blank. The absorptions were determined using molar extinction coefficient (molar absorptivity) as formed MDA in terms of μmol/mg of protein (14).

**Measuring the concentration of reduced glutathione (GSH)**

Glutathione was measured using Rahman et al. method. That is, 25 μl of serum sample or homogenized tissue was poured into ELISA microplates and 140 μl of Tris-EDTA 0.2 M buffer with PH=8 was added to it. Finally, 30 μl of DNTB 0.1 M was added and mixed. The absorption was determined using an ELISA reader device at a wavelength of 412 nm in wells. The absorptions were determined using molar extinction coefficient (molar absorptivity) as formed glutathione in terms of μmol/mg of protein (15).

**Measuring the activity of catalase (CAT)**

Catalase was measured using Aebi et al. method. That is, 1000 μl of 50 mM potassium phosphate buffer with PH=8 and 50 μl of serum sample or homogenized tissue was added to a tube. Finally, at the time of reading the amount of absorption of
the samples by a spectrophotometer, 50 μl of H2O2 was added to the mixture and sample absorption was measured against a reagent blank after 0, 30 and 60 seconds at a wavelength of 240 nm. The absorptions were determined using molar extinction coefficient (molar absorptivity) as catalase activity in terms of enzyme unit per mg of protein (16).

**Glutathione peroxidase activity (GPX)**
Glutathione peroxidase was measured using the Rotruck et al. method. That is, 200 μl of Tris-HCl 0.4 M buffer with PH=7, 100 μl of sodium azide 1 mM, 200 μl of serum sample or homogenized tissue, 200 μl of Glutathione 2 mM, and 100 μl of hydrogen peroxide 0.2 mM was added to a tube and incubated for 10 minutes at 37 °C. Then, 0.4 ml of TCA 10% was added to the tube and centrifuged for 3 minutes at 2000 RPM. Then, 25 μl of the supernatant inside the tube was poured in ELISA microplates and 140 μl of Tris-EDTA 0.2 M with PH=8 and 30 μl of DNTB was added to it. After 30 minutes of incubation at room temperature, absorption of samples was determined using ELISA reader device at 420 nm. The absorptions were determined using molar extinction coefficient (molar absorptivity) as glutathione peroxidase activity in terms of enzyme unit per mg of protein (17).

**Data statistical analysis**
Results are presented as mean±standard deviation. The difference between the mean lipid peroxidation and antioxidant enzyme activity among different groups was estimated using one-way ANOVA followed by Duncan's multiple range test. The statistical significance level was considered as P<0.05.

**Results**
Figure 1 shows the results of catalase activity among different groups. Catalase activity showed a significant decrease in the control group compared to the PPHE200, PPHE250, and Vit E groups (P<0.05). The enzyme activity had a significant difference between the Vit E and PPHE200 groups (P<0.05), while the difference was not significant between Vit E and PPHE250 groups (Figure 1).

Figure 2 shows the results of glutathione peroxidase activity among different groups. Serum glutathione peroxidase activity showed a significant decrease in the control group compared to the PPHE200, PPHE250, and Vit E groups (P<0.05). The enzyme activity had a significant difference between the Vit E and PPHE200 groups (P<0.05), while the difference was not significant between Vit E and PPHE250 groups (Figure 2).

Figure 3 shows the results of the amount of reduced glutathione among different groups. Serum reduced glutathione amounts showed a significant decrease in the control group compared to the PPHE200, PPHE250, and Vit E groups (P<0.05). In the means comparison test, the amount of reduced glutathione among the Vit E, PPHE200, and PPHE250 groups were significantly different (P<0.05), while the difference was not significant between PPHE200 and PPHE250 groups (Figure 3).

Figure 4 shows the results of the amount of MDA among different groups. Serum MDA levels showed a significant increase in the control group compared to the PPHE200, PPHE250, and Vit E groups (P<0.05). In the means comparison test among the Vit E, PPHE200 and PPHE250 groups the difference was not significant (Figure 4).
Table 1: Activity of catalase, glutathione peroxidase, and levels of reduced glutathione and MDA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experimental treatment</th>
<th>One 1</th>
<th>Two 2</th>
<th>Three 3</th>
<th>Four 4</th>
<th>SD</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Catalase (units/mg protein)</td>
<td></td>
<td>2.58±0.38</td>
<td>4.38±0.45</td>
<td>3.57±0.24</td>
<td>3.6±0.59</td>
<td>0.28</td>
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<td>Glutathione peroxidase</td>
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<td>76.72±4.49</td>
<td>97.61±5.91</td>
<td>105.63±2.88</td>
<td>c110.01±8.36</td>
<td>4.76</td>
<td>0.000</td>
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<tr>
<td>(units/mg protein)</td>
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<td></td>
<td></td>
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<tr>
<td>Glutathione (µmol/mg</td>
<td></td>
<td>75.26±1.35</td>
<td>89.39±6.98</td>
<td>93.54±3.72</td>
<td>107.86±4.76</td>
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<tr>
<td>MDA (µmol/mg protein)</td>
<td></td>
<td>5.88±0.56</td>
<td>3.62±0.15</td>
<td>3.35±0.51</td>
<td>3.26±0.25</td>
<td>0.25</td>
<td>0.000</td>
</tr>
</tbody>
</table>

1. Rats receiving physiologic serum (control); 2. Rats receiving 200 mg/kg of hydroethanolic extract of pomegranate peel; 3. Rats receiving 250 mg/kg of hydroethanolic extract of pomegranate peel; 4. Rats receiving 5 mg/kg of vitamin E.

Figure 1: Catalase activity among the experimental groups:

\*\* The column with dissimilar letters has statistically significant differences (P<0.05).

Figure 2: Glutathione peroxidase activity among the experimental groups:

\*\* The column with dissimilar letters has statistically significant differences (P<0.05).
Figure 3: The amounts of reduced glutathione among the experimental groups:

\(^{a-c}\) The column with dissimilar letters has statistically significant differences \((P<0.05)\).

Figure 4: The amounts of MDA among the experimental groups:

\(^{a-c}\) The column with dissimilar letters has statistically significant differences \((P<0.05)\).

**Discussion**

Antioxidant enzymes and antioxidant compounds (non-enzymatic) change toxic free radicals to non-toxic products and thereby protect cells from damage caused by oxidative stress \((18, 19)\). Polyphenolic compounds in pomegranate peel might account for the antioxidant and anti-cancer effects of its extract \((20)\). Therefore, it can be suggested that the antioxidant activity of the hydroalcoholic extract of pomegranate peel was due to the presence of these compounds. In addition, vitamins E is essential for normal functioning of the body cells during exercise and it has been shown that the production of free radicals and lipid peroxidation in liver and skeletal muscle increased significantly in rats with vitamin E deficiencies under the stress of intensive exercise \((21)\). Since all chemical reactions in cells are under the precise control of enzymes, unwanted side reactions such as lipid peroxidation and protein oxidation need to be minimized for the normal functioning of cells and compliance and control of metabolism in the body. This depends to a large extent on maintaining the natural structure of the molecules against metabolic disorders. However, the production of superoxide radicals \(\text{O}_2^{\cdot -}\) and hydroxyl (OH) due to physical activity is
inevitable, resulting in the production of MDA and attacks on cell membrane lipids (22). Therefore, the present study examined the potential synergic impact of using antioxidants with external sources along with regular exercise of swimming, followed by the stress of intensive exercise. The results showed that the oral use of a hydroalcoholic extract of pomegranate peel and vitamin E as a source of antioxidants, along with regular exercise can reduce the effects of stress from intensive physical activity, and enhance antioxidant defense system of the body.

The results showed that the consumption of 200 mg/kg hydroalcoholic extract of pomegranate peel and 250 mg/kg of vitamins E in the treatment groups reduced serum lipid peroxidation compared to the control group. Exhaustive sports directly increased lipid peroxidation in the plasma through the inhibition of the enzymatic defense system of red blood cells (23, 24) and many studies have reported increased concentrations of MDA as an indicator of lipid peroxidation in people after a session of exhaustive exercise (25, 26). Ji et al. reported that after a session of acute physical exercise, the concentration of MDA increased in rats (24). In another study, Alessio et al. reported lipid peroxidation increase during exhaustive exercise (27). In another study, consumption of pomegranate peel methanol extract reduced serum MDA (28). Similarly, consumption of ginseng extract reduced the amount of serum MDA in rats under exhaustive exercise stress (29).

Another study showed that the consumption of pomegranate peel methanol extract decreased lipid peroxidation in the liver, heart, and kidney (30), which is consistent with the results of this study. It appears that the ability of the hydroalcoholic extract of pomegranate peel and vitamin E in the reduction of oxidant molecules resulted from the inhibition of reactive oxidative species (ROS) leading to lipid peroxidation reduction (31).

The present study showed that the consumption of 200 mg/kg of hydroalcoholic extract of pomegranate peel and 250 mg/kg of vitamins E in the treatment groups increased the amount of reduced glutathione compared to the control group. Previous studies have shown that exhaustive exercise causes oxidative damages and oxidation of glutathione (32, 33, 34). In a study on marathon runners, the amount of plasma oxidized glutathione increased immediately after a marathon (32). Although the results of a study suggest that the consumption of pomegranate peel methanol extract increased the serum levels of glutathione (28), in another study, the consumption of pomegranate juice along with regular exercise caused changes in serum levels of reduced glutathione compared to the control group (35). The results showed that the extract of Hypoestes phyllostachya enhanced the serum levels of reduced glutathione (36) which is consistent with the results of this study. It appears that the consumption of pomegranate peel hydroalcoholic extract and vitamin E reduces the deterrent effect of the glutathione synthesis inhibiting enzyme (36).

The results showed that the consumption of 200 mg/kg of hydroalcoholic extract of pomegranate peel and 250 mg/kg of vitamins E in the treatment groups increased glutathione peroxidase activity in serum compared to the control group. The results of previous studies have shown that glutathione peroxidase activity under exhaustive exercise stress decreased in muscle tissue and serum (32, 37) or did not
The effect of pomegranate peel hydroalcoholic extract on the activity of antioxidant enzymes in the serum and muscle tissues of rats under exhaustive exercise stress: A comparison of the effects of pomegranate peel methanol extract and vitamins E.

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The consumption of methanol extract of cinnamon led to a non-significant increase in glutathione peroxidase activity in muscle tissue of rats treated with the extract under exhaustive exercise stress compared to the control group (40). However, Voces et al. reported that ginseng extract consumption improved liver antioxidant conditions by glutathione peroxidase activity (41). Similarly, in another study, pomegranate peel methanol extract increased the activity of this enzyme in the brain of rats (28) which is consistent with the results of the present study. The present study showed that the consumption of 200 mg/kg of hydroalcoholic extract of pomegranate peel and 250 mg/kg of vitamins E in the treatment groups increased catalase activity in serum compared to the control group. Previous studies have shown that the consumption of pomegranate peel methanol extract increased catalase activity in the brain of rats (28), and in another study, ginseng extract increased catalase activity in muscle of rats under acute exercise stress (42), which is consistent with the results of the present study. It appears that the inhibitory power of the hydroalcoholic extract and vitamin E in neutralizing free radical oxygen species contribute to the enzymatic immune system leading to increased activity of glutathione peroxidase and catalase enzymes.

Conclusion
According to the results, pomegranate peel hydroalcoholic extract and vitamins E as external sources of antioxidants can neutralize free radicals and help the enzymatic immune system to reduce the effects of oxidative stress due to intensive physical activity.

Conflict of Interest
The authors expressed no conflict of interests.

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