Effect of different loads of exercise and *Nigella sativa* L. seed extract on serologic and hematologic parameters in rat

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There are many recommendations for prophylactic usage of *Nigella sativa* L. for healing fatigue and body strengthening. However, a scientific and standard method for diagnosis and management of overtraining in athletes has not been introduced. Here, we studied the effect of different loads of exercise and *N. sativa* treatment on serologic and hematologic parameters in rat was examined. Male Wistar rats were divided into control sedentary (C), moderate trained (MT), overtrained (OT), control sedentary + *N. sativa* (NC), moderate trained + *N. sativa* (NM) and overtrained + *N. sativa* (NO) treatment. Animals’ performances were evaluated before and during the study. Immediately, 24 h and two weeks after the last bout of exercise serum concentration of corticosterone and lactate as well as total and differential WBC, RBC, Hb, Hct, corpuscular indices and platelets were also measured. Increasing training load caused a significant performance decline in OT and OR groups (*P* ≤ 0.01- ≤ 0.001). Compared to control group, serum corticosterone and lactate concentrations were significantly increased after moderate exercise and overtraining (*P* ≤ 0.05 to ≤ 0.001), total WBC count (*P* 0.001), lymphocytes (*P* <0.01) and monocytes (*P* ≤ 0.01) were decreased in overtraining animal. *N. sativa* treatment caused a significant decrease in lactate concentration in overtraining (*P* <0.01) and serum corticosterone in all exercised (*P* ≤ 0.05) compared to untreated groups. Overtraining induced chronic inflammatory like changes, performance decline, stress hormone elevation, and WBC count decrement. *N. sativa* administration improved corticosterone elevation and metabolic state.

**Keywords:** Black cumin, Black seed, Corticosterone, Fatigue, Fennel flower, Herbal, Lactate, Hematological factor, Overtraining syndrome

Sedentary lifestyle is a major risk factor for different chronic pathologies which increases the morbidity and mortality of many chronic non-communicable diseases like type 2 diabetes, cancer, cardiovascular and neurodegenerative diseases⁴. Regularly performed endurance exercise with moderate intensity and duration has many health benefits like reduction of systemic low-grade inflammation and protects the body against chronic diseases²³.⁵

In competitive training excessive overload in combination with inadequate recovery can lead to overreaching and possibly overtraining. However, the time needed to recover from overtraining syndrome is considered to be much longer (months to years)⁴⁵. In overtraining, the homeostatic balance involving a wide range of hormonal, metabolic, and immunologic factors is altered²³⁵. The imbalance between training period and recovery may lead to functional over-reaching (FOR), non-functional overreaching (NFOR) or overtraining syndrome (OTS). The boundary between NFOR and overtraining syndrome is difficult to be determined in human⁶. The severe fatigue and under performance period may last several months to several years in OTS but few weeks to several month in NFOR⁴. A scientific and standard method for diagnosis and management of overtraining in athletes is still lacking. Hence, we made an attempt to study the effects of enforced physical exercise, especially with different loads, on hematological and serological parameters.

Therapeutic effects of herbal medicines, including antioxidant, anti-inflammatory, anticancer, antimicrobial and immunomodulatory have been demonstrated by many researchers earlier⁷-¹². Black seed or *Nigella sativa* L. (Fam. Ranunculacea) is one such historically well-known herb¹¹. In the “Canon of medicine”, Avicenna recommended *N. sativa* as a herbal remedy to stimulate the body energy and ameliorate fatigue and depression¹³. In addition, *N. sativa* therapeutic potential in a wide range of illnesses had
been documented\textsuperscript{14}. Antioxidant, anti-inflammatory, antimicrobial, antitumor, antitussive and immunomodulatory properties of \textit{N. sativa} and its ingredients\textsuperscript{14-16} have been shown. Similarly, the protective effect of \textit{N. sativa} against toxicity of carbon tetrachloride\textsuperscript{17}, sulfur mustard\textsuperscript{18} and hematological parameters\textsuperscript{19} apart from the anti-inflammatory effects of \textit{N. sativa} and its constituents in several diseases, such as experimental allergic encephalomyelitis, colitis, and arthritis\textsuperscript{11} as well as sensitized animals\textsuperscript{20}, asthmatic patients\textsuperscript{21,22} and chemical war victims\textsuperscript{23} have been demonstrated already.

Prophylactic usage of the \textit{N. sativa} for healing fatigue and body strengthening is also being recommended in several studies\textsuperscript{13,24}. In this study, we investigated the effect of ethanolic extract of \textit{Nigella sativa} seeds on serologic and hematological changes due to exercise, moderate and heavy, using rat model.

**Material and Methods**

**Preparation of extract**

\textit{N. sativa} seeds were purchased from a local herbal store and authenticated by botanists in the herbarium of Ferdowsi University of Mashhad (specimen number: 293-0303-1). The macerated hydroethanolic extract was prepared by mixing 200 g of chopped \textit{N. sativa} seeds with 800 mL of 50\% ethanol for 72 h at 40°C. The solutions after filtration were dried by rotary evaporation at 40°C. This process was repeated thrice during the study.

**Animals**

Fifty adult (6-8 week old) male Wistar rats, (Animal House of School of Medicine, Mashhad University of Medical Sciences, Mashhad, IR Iran) weighing 150-200 g were housed under 12 h light/dark cycle; 22-24°C and food and water were available \textit{ad libitum} throughout the experiment. Animals were allowed to adjust to new condition for two weeks. The protocols used conformed to guidelines of the conduct animal studies, were approved by the committee on the ethics of animal experiments in Mashhad University of Medical Sciences.

**Training Protocol**

A motorized treadmill with 4 individual lanes with a shock grid at the back of the treadmill provided a mild shock (0.5 mA, 1 HZ) was used. The animals were undertaken a familiarizing period. They were placed on the treadmill 5 days for 10 min/day at a speed of 12 m/min at 0\% degree inclination and were scored 1-5 depending on running quality. To exclude possible different levels of stress between animals, rats that run with mean rating 3 or higher (\textit{n}=42) from those refused (\textit{n}=8) had were chosen for the study\textsuperscript{5}. Trainable animals were randomly assigned to experimental groups. During the experimental period, different groups of animals had been maintained and treated in similar condition during the study avoiding other stress condition than training load and physical stress.

Seven groups of animal including: control sedentary (C), moderate trained (MT), overtrained (OT), recovered overtrained (OR), control sedentary + \textit{N. sativa} (NC), moderate trained + \textit{N. sativa} (NM) and overtrained + \textit{N. sativa} (NO) were studied (\textit{n}=6 for each group). Treated animals received drinking water containing 200 mg/kg/day of \textit{N. sativa} extract during the training time period. The animals of the both control groups (treated and untreated) were handled and placed on the treadmill to experience the stress of treadmill environment. Exercised groups undertook a progressive load training 6 days a week to enhance cardiorespiratory fitness and a 5 min warm up and cool down were done each session. Moderate trained groups underwent 8-week exercise at a speed of 15 m/min for 20 min, 6 days/week but the intensity of exercise was increased to 20 m/min for 30 min at the onset of the second week (Table 1).

<table>
<thead>
<tr>
<th>Experimental week</th>
<th>Performance test</th>
<th>Training phase</th>
<th>Training speed (m/min)</th>
<th>Training Time (min)</th>
<th>Number of daily session</th>
<th>Recovery between session (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT/OT</td>
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<td>MT/OT</td>
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<td>OT</td>
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<td>1</td>
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<td>15</td>
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<td>-</td>
<td>1</td>
<td>20</td>
<td>22.5</td>
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<td>45</td>
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<td>4</td>
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<td>3</td>
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<td>30</td>
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<td>5</td>
<td>3</td>
<td>20</td>
<td>25</td>
<td>-</td>
<td>60</td>
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<td>10</td>
<td>6</td>
<td>3</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>60</td>
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<td>11</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1—Training protocol of moderate group (MT) and overtraining group (OT)
Overtrained groups were submitted into three-phase program. In the first 4 weeks (phase I) training, speed was increased from 15 to 25 m/min and training time from 20 min to 60 min. In the second 4 weeks (phase II), training load was maintained constant. During last 3 weeks (phase III), running intensity and training duration remained unchanged but recovery time between training sessions was reduced (from 24 h to 4, 3 and 2 h) (Table 1). The OR group had two week recovery period after the last exercise session\(^{11}\). The training program was evaluated by performance test at the end of 0, 2, 4, 8 weeks for MT group and continued to 8-11 weeks for OT and also after two weeks recovery for OR group.

In performances test, the animals’ overexhaustion were determined by increasing the speed of treadmill gradually (1 m/min every 2 min till speed of 20 m/min, then 2 m/min every 3 min until animals refused to run). A mass model (mass×speed stage×minutes performed at each stage) was used for performance quantification (Kg.m) and determination of animals decline in performance and over exhaustion\(^5,25\).

**Sample collection**

At the end of the study, animals were anesthetized with diethyl ether and peripheral blood was collected from the retro-orbital sinus in the control group, immediately and 24 h after the last session of exercise in MT, OT, NC, NM and NO groups and immediately, 24 h and 2 weeks after the last session of exercise in OR group. Samples collection was done at 09.00 a.m. for all groups.

A sample of blood was collected in EDTA tube and was analysis of routine CBC including total and differential WBC and platelet counts. For lactate measurement; blood was transferred to 1.5 mL tubes containing 50 μL sodium fluoride (1%) and its concentration was determined by enzymatic method (Ziest Chem kit, Iran, Tehran). For measurement of corticosterone, blood was clotted, serum samples were separated by centrifuging at 3000 rpm for 10 min, was collected and stored at −20°C, and corticosterone analyzed by Elisa method (abcam kit, UK, Cambridge).

**Statistical analysis**

The results were presented as means ± SEM. Two group comparisons were done using unpaired "t" test. Comparisons of more than two groups were performed using a one way analysis of variance (ANOVA) with Tukey-Kramer post-tests. Within groups comparison (different time) was done using repeated ANOVA. Significance was accepted at \( P < 0.05 \).

**Results**

**Comparisons of performance between groups**

The MT animals showed a significant increase (\( P < 0.001 \)) in performance at test 3 to 4 compared with test 1. In OT and OR groups, animal performance increased (\( P < 0.01 \) to \( P < 0.001 \)) during first 8 weeks but increasing training load caused a significant decline (\( P < 0.001 \)) in next three weeks. Even after 2 weeks recovery in OR group, performance was lesser (\( P < 0.001 \)) than pre-overload value. *N. sativa* administration in NM and NO groups had no significant effect on improving performance test results (Table 2).

**The effect of *N. sativa* and exercise on serum concentration of lactate and corticosterone**

Lactate concentrations increased significantly after moderate exercise and overtraining (\( P < 0.05 \) for MT and \( P < 0.001 \) for OT) compared to the control group. It was also significantly higher in OT than MT group (\( P < 0.001 \)). In NO group, there was a significant decrease in lactate concentration compared to OT (\( P < 0.01, \text{Fig. 1} \)).

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**Table 2**—Performance Test (PT, Kg.m) analysis in Moderate trained (MT), overtrained (OT), recovered overtrained (OR), groups (for each group, n=6)

<table>
<thead>
<tr>
<th>Week</th>
<th>PT</th>
<th>MT</th>
<th>OT</th>
<th>OR</th>
<th>NM</th>
<th>NO</th>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>174.03±13.12</td>
<td>135.52±17.1</td>
<td>140.69±15.99</td>
<td>182.35±19.99</td>
<td>173.52±21.31</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>258.17±37.18</td>
<td>308.21±31.15***</td>
<td>281.36±26.51***</td>
<td>197.11±24.06</td>
<td>187.12±23.03</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>337.42±42.54***</td>
<td>343.42±36.8***</td>
<td>337.93±25.74***</td>
<td>235.72±24.08</td>
<td>325.18±41.47**</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>431.2±34.69***</td>
<td>449.52±38.1***</td>
<td>446.15±43.67***</td>
<td>386.89±12.02***</td>
<td>413.9±27.26***</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>-</td>
<td>260.49±31.44***</td>
<td>227.76±24.47***</td>
<td>-</td>
<td>276.37±15.15***</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>-</td>
<td>200.13±30.9***</td>
<td>127.16±19.86***</td>
<td>-</td>
<td>143.05±15.17***</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>-</td>
<td>146.67±16.06***</td>
<td>112.74±17.07***</td>
<td>-</td>
<td>132.63±19.27***</td>
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<tr>
<td>13</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>114.95±14.3***</td>
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</table>

[Values are presented as mean ± SEM. Statistical differences between PT1 and PT2, PT3, PT4: * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \). Statistical differences between PT4 and PT5, PT6, PT7, PT8: ** \( P < 0.01 \); *** \( P < 0.001 \). Data of each test in groups was compared with PT1 or PT4 using Repeated Measures Analysis of Variance]
Immediately after the last bouts of exercise, serum corticosterone significantly increased in both MT and OT groups compared to the control group ($P < 0.01$ for both cases, Fig. 2) which decreased significantly in 24 h and 2 weeks group after last bouts of exercise ($P < 0.05$ and $P < 0.001$, respectively), however, it didn't return to control level (Fig. 1). In *N. sativa* treated groups, serum corticosterone was significantly higher in only NO than NC group ($P < 0.01$). *N. sativa* treatment in exercised groups (NM and NO) also showed significantly decreased serum corticosterone compared to the untreated groups ($P < 0.05$ for both cases, Fig. 1).

**Blood variable comparison before and after exercise**

Only eosinophil percent was found increased after moderate exercise ($P < 0.05$). Total WBC ($P < 0.001$), lymphocytes ($P < 0.001$), neutrophils ($P < 0.05$) and monocytes ($P < 0.01$) were significantly lower immediately after last bout of overtraining compared to pre-exercise values. After two weeks recovery, total WBC count was restored, while lymphocytes ($P < 0.05$) and monocyte percentage ($P < 0.05$) remained significantly less than pre-exercise value. Two weeks recovery caused a significant increase in total WBC ($P < 0.05$) and total lymphocyte count ($P < 0.05$) in comparison to the immediately after exercise values. Immediately after overtraining, MCV ($P < 0.01$) and MCH ($P < 0.05$) decreased and MCHC ($P < 0.01$) increased compared to pre-training values. After two weeks recovery, the MCV index decreased ($P < 0.05$) compared to the pre-training value (Table 3).

**Comparison of blood variables in MT, OT and control groups, immediately after exercise**

Overtraining induced a significant decrease in the total WBC count ($P < 0.001$), lymphocytes ($P < 0.01$) and monocytes ($P < 0.01$) but percent of eosinophils increased significantly in MT ($P < 0.05$) and OT ($P < 0.01$) groups compared to control. Only monocytes absolute count was lower in OT compared to MT group ($P < 0.05$). In addition, only MCHC was lower in MT ($P < 0.01$) and OT ($P < 0.001$) compared to control group (Table 4).

**Comparison of blood variables between MT, OT and control groups, 24h after exercise**

There was no statistical difference in total and differential WBC count between MT and OT groups; and only absolute monocytes count was lower in OT in comparisons to MT group ($P < 0.01$). In addition, there were no significant differences in values of platelet, RBC, Hb, Hct, RBC indices between MT and OT groups (Table 4).

**Comparison of blood variables in NC, NM and ON groups, immediately after exercise**

In NO group, the total WBC count, total lymphocytes and neutrophils count decreased significantly compared to the NC ($P < 0.001$) and NM ($P < 0.05$) groups. The Hb values ($P < 0.05$), MCH ($P < 0.05$) and MCHC ($P < 0.01$) indices were significantly lower in NO than NC groups. In addition
the MCH (P < 0.05) and MCHC (P < 0.05) indices were significantly lower in NO than NM groups (Table 4).

Comparison of blood variables in NC, NM and ON groups, 24 h after exercise

Monocytes count was lower in NO than NC group (P < 0.05). In NO group the absolute lymphocyte (P < 0.01) and monocytes (P < 0.05) count was statistically decreased compared to NM group (Table 4).

Blood variable comparisons in treated and untreated animal

Only Hb (P < 0.05) and MCV (P < 0.05) values were statistically higher in NM group than MT group. Eosinophils count was lower in NC than untreated control group (P < 0.05). The RBC count (P < 0.01) was higher but the MCV (P < 0.05) and MCH (P < 0.05) indices were statistically lower in NC group than untreated control group. Neutrophils percentage was lower in NO than OT group (P < 0.05). In addition, MCH (P < 0.05) and MCHC (P < 0.05) indices were also lower in NO than OT group (Table 4).

Discussion

We used a standard training protocol for overtraining with an imbalance period between exercise bouts and rest in third phase. Therefore, despite the performance improvement in first 8 week in all training groups, it caused overtraining symptoms like fatigue, lethargy, and decline in performance which last even after two weeks recovery in OR groups. Overtraining syndrome is defined as the accumulation of intensifying training stress without enough recovery, which has resulted in several weeks or months performance decline. The present study overtraining protocol was able to reduce animals' performance as a main marker of overtraining syndrome. Other findings such as higher lactate level in OT group in comparison to MT group, also suggested increase in corticosterone levels in OT group compared with control group could have contributed to the observed decrease in performance. Maximal lactate steady state (MLSS) has been proposed as a standard method for anaerobic and exhaustion threshold determination. In rat treadmill running using continues protocol, the MLSS was reached at a velocity of 20 m/min and at 25 m/min the blood lactate level was increased progressively. A significant correlation between lactate levels during severe exercise training and muscle fatigue had been suggested. The rapid increase in blood and muscle lactate (due to tissue hypoxia) can lead to muscular fatigue, sympathetic stimulation and so increased in cardio-vascular responses. Overtraining under-performance is thought to be associated with an increase in lactate level and muscle glycogen depletion, which is linked to ROS

![Table 3—Comparison of blood variable before and after exercise in moderate trained (MT), overtrained (OT), recovered overtrained (OR) groups](image-url)
generation and the imbalanced antioxidant defense system. Moreover, exercise induced ROS generation effects are important in initiating adaptive processes as well as reducing the damaged enzymes and cell damage\(^3,28\). Retrieving glycogen reserve in muscle tissue, blocking the muscle inflammatory reaction and preparing the organism for the next bout of exercise are cortisol roles in exercise. Stress hormones (glucocorticoids and catecholamines) marked elevation after sever exercise could be a part of body response to its metabolic disturbance and catabolic states\(^3,29\). But repeated exposure to stress can lead to changes in the releasing pattern and sensitivity to those hormones\(^3,30\). Glucocorticoids elevation after acute and chronic exercise can resulted in lower sensitivity and adaptation of HPA axis and other cells and tissues (blood monocytes) to hormone or its receptor\(^29,31,32\). Moreover, there is a close correlation between cytokine production and cortisol concentration\(^3,33\). As our previous study result, there is a marked increase in IL-6 concentration following overtraining\(^3,34\) which can stimulate cortisol secretion\(^3,34\).

In this study, overtraining but not the moderate exercise caused a significant decrease in total WBC and lymphocytes, neutrophils and monocytes counts in comparison to before exercise and control group values. Although leucocytosis due to neutrophilia is one of the most consistent changes which have been observed during and after acute exercise\(^3,35\) but there are few exception for chronic exercise\(^3,36\). Johannsen et al.\(^3,36\) reported a significant reduction in total WBC and neutrophil counts after aerobic exercise levels at or
above public health recommendation in postmenopausal women. It was showed that the impact of aerobic and anaerobic exercise on hematological variables were mostly similar

Several mechanisms exist that may explain the reduction in total WBC, and specially neutrophils, counts with exercise training.

Exercise may alter the trafficking of leukocyte subsets between secondary lymphoid organs and blood. In addition, the possibility that exercise directly impacts bone marrow hematopoiesis and the related changes in WBCs in blood need further investigation. The IL-6 release following exercise was associated with neutrophil mobilization and priming for oxidative activity. In addition, it is believed that IL-6 and adiponectin concentration may be the most effective agent in correcting total WBC count by independently lowering lymphocytes, monocytes, and basophils number.

Although the Hb and Hct values differences between trained and untrained animals or pre and post exercise was not significant. The RBC count increased significantly after two weeks recovery in OR group; and also MCV and MCH indices decreased while MCHC increased significantly in OT group immediately after exercise in comparison to pre exercise values. These minimal changes may be due to the effect of tissue hypoxia and the corticosterone increment during exercise on RBC counts and the effect of exercised induce plasma shifting on Hb/Hct ratio. Other studies reported no change in RBC count, Hct and Hb in marathon runners and reduced Hb in overtraining rats.

Little information is available about *N. sativa* tonic properties on exercise. Our previous study result showed that chronic administration of *N. sativa* may change pro and anti-inflammatory cytokines profiles, and may act as a balancing factor on Th1/Th2 lymphocytes in different exercise loads while may act as an inhibitory factor on Th2 phenotype in control animals. In this study, *N. sativa* treatment could not improve performance decrement in overtrained group. In previous studies, treatment with other supplements such as L-arginine had no significant effect on VO2 max decline and fatigue of high intensity exercise, but it reduced end exercise blood lactate accumulation

In this study, *N. sativa* treatment caused a significant decrement in lactate concentration of overtraining group. This could be due to its volatile oil and protein components effects on metabolism, liver enzymes and glycolysis pathway like hexokinase or its modulating effect on NO production. *N. sativa* treatment also significantly decreased corticosterone concentration in both moderate and overtraining exercised groups. This could approve the *N. sativa* modulating effects on metabolism and body response to stressogenic condition. In addition, *N. sativa* has a modulating effect on NO production and it had been shown that NOS activity modulates the response of the neuroendocrine component of the HPA axis during exercise stress.

*N. sativa* treatment in MT and OT groups did not show any significant effect on hematological values in comparison to untreated groups. However, *N. sativa* treatment in control group significantly increased RBC count and decreased MCV and MCH indices. It was reported that using *N. sativa* in the normal rabbits significantly increased platelet count but did not have any effect on other blood factors WBC, RBC, Hb, Hct. Most of the previous studies evaluated the *N. sativa* protective effect against toxins, such as carbon tetrachloride, diazinon, cadmium, sulfur mustard and other toxins or its preventive effects in various chronic illnesses including cancerous, diabetic, cardiovascular, pulmonary, neurological and autoimmune diseases. Our present study yielded mixed results on the beneficial aspects of *N. sativa* seed extract after exercise.

**Conclusion**

According to the present study findings, overtraining induced chronic inflammatory symptoms, such as lethargy, fatigue, performance decline, stress hormone elevation, catabolic/anabolic imbalance and WBC count decrement. Measurement of testosterone/corticosterone ratios and ROS generation would help to elaborate catabolic/anabolic imbalance and proinflammatory status after overtraining and physiological compensation during the recovery period. Long term administration of *Nigella Sativa* though improved some changes like corticosterone elevation and metabolic state, it did not show any significant effect on hematological factors.

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References


