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Protective effects of lupeol on acute exercise-related oxidative stress in male rats

Emrah ÖZDEMİR [1], Hakkı ÇOKNAZ [2]*, Sıla Özlem ŞENER [3], Ufuk ÖZGEN [4], Ali GÖK [5]

1 İstanbul Rumeli University, Faculty of Sport Sciences, 34744 İstanbul, Türkiye.
2 Düzce University, Faculty of Sport Sciences, 81620 Düzce, Türkiye.
3 University of Health Sciences, Faculty of Pharmacy, 06018 Ankara, Türkiye.
4 Karadeniz Technical University, Faculty of Pharmacy, 61080 Trabzon, Türkiye.
5 Experimental Animals Laboratory of Düzce University, 81620 Düzce, Türkiye.
* Corresponding Author. E-mail: hakkicoknaz@duzce.edu.tr (H.Ç.); Tel. +90-533-381 07 70.

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ABSTRACT: The aim of this study is to determine the protective effects of lupeol on acute exhaustive exercise-induced oxidative stress in male rats. Rats were randomly selected and divided into four groups (n=6/group): Control, exercise, lupeol and exercise+lupeol groups. At the end of the exercise protocol, the rats were sacrificed by cervical dislocation, blood samples were taken. The levels of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH), coenzyme Q10 (CoQ10) and α-lipoic acid (ALA) and oxidative stress levels in serum samples were measured. As a result, it has been determined that lupeol, a natural triterpenoid compound, contributes positively to the level of antioxidants produced by the body and helps suppress oxidants. It has been observed that free radicals caused by oxidative stress that occur as a result of high-intensity exercise are reduced by lupeol. Accordingly, it has been determined that the use of lupeol during exercise in experimental animals exposed to oxidative stress inhibits free radicals formed by stress. In the light of these results, it is thought that lupeol may be included in the composition of pharmaceutical products against exercise-induced oxidative damage.

KEYWORDS: Antioxidant; exercise; free radical; lupeol; oxidative stress.

1. INTRODUCTION

Exercise are planned activities to improve physical performance such as muscle strength, reaction time, neuromuscular system, balance, aerobic and anaerobic capacity [1]. Energy expenditure of the body through skeletal muscles is called physical activity. During physical exercise, the metabolic rate increases proportionally with the increase in the intensity of muscular activity [2]. Physical exercises can cause oxidative stress based on severity and duration. In relation to this, lipid peroxidation is thought to occur if the rise in the level of free radicals at the time of exercise passes antioxidants in the defense capacity of the cells [3]. Increased consumption of O_2 during exercise leads to increased free radical formation [4].

It can be thought that the extent of damage to the body will have effects on the regeneration process in athletes. But exercise of certain severity and regularity is known to strengthen antioxidant defense [5].

It has been revealed that acute exercise will cause oxidative stress, especially when performed with high severity. There are two systems associated with oxidative stress in acute and aerobic exercise as followed: When VO_2 rest levels increase 10-15 times, prooxidant activity increases with the effect of mass event and antioxidant activity is stated to be insufficient compared to prooxidants [6].

These emerging free radicals are neutralized through a defense mechanism that covers enzymatic and nonenzimatic antioxidants. It is stated that an imbalance defined as oxidative stress between exercise and antioxidants causes the emergence [4]. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) are cause enzymatic defense. Nonenzimatic antioxidants are glutathione, melatonin, uric acid, bilirubin, albumin, coenzyme Q10, α-lipoic acid, selenium, seruloplazmin and transferrin [7].

Since oxidative damage of our cells increases with age, the increased intake of exogenous antioxidants from fruit and vegetables may support the endogenous antioxidative defense. The antioxidants such as

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vitamin C and vitamin E, carotenoids, and phenolic compounds (e.g., flavonoids, catechins) are presently considered to be the powerful exogenous antioxidants. Clinical studies imply that eating a diet rich in fruits, vegetables, wholegrains, legumes, and omega-3 fatty acids can help humans in disease prevention [8].

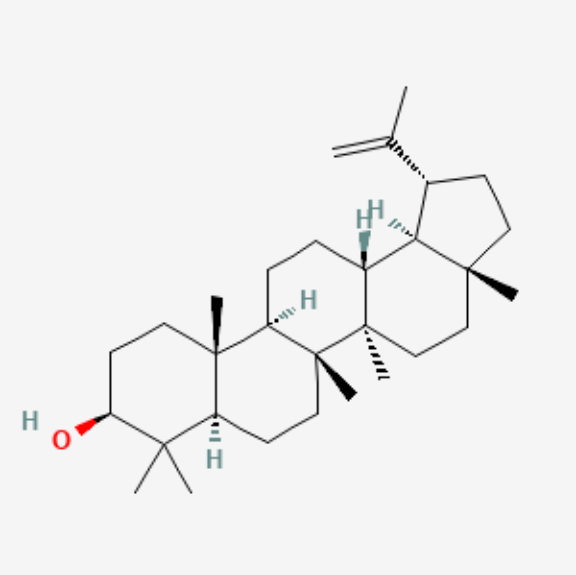

Figure 1. Lupeol

Lupeol is a natural compound in a lupane-type triterpene structure (Figure 1). It is especially represented in the plants, but rare in fungi and animal kingdoms [9]. Lupeol is found in vegetables such as white cabbage, pepper, cucumber, tomato; fruits such as olives, figs, mangoes, strawberries, red grapes, and some medicinal plants such as American ginseng, Shea butter plant, *Tamarindus indica*, *Allanblackia monticola*, *Cirsium* species [10, 11]. Lupeol is a pharmacologically active compound as well. It stands out with its important activities such as antioxidant, anticancer and anti-inflammatory activity, but also has several potential medicinal properties [12]. Looking at the literature, studies on determining the protective effects of lupeol on acute exhaustive exercise-related oxidative stress in male rats have not been found. In this study, it is intended to demonstrate the effects of acute exhaustive exercise on oxidant stress and the extent to which lupeol prevents oxidative damage during exercise.

2. RESULTS

The results of the study have been given in Table 1.

When Table 1 is examined, **Control group,** it was evaluated for SOD 85.55±10.81(U/mL), CAT 11.78±1.97 (ng/mL), MDA 13.03±5.00, GSH 13.23±15.87 (µg/mL), CoQ10 4.39±1.84 (ng/mL), ALA 4.98±0.02 (µg/mL), TAS 0.73±0.10 (mmol/L), TOS 7.75±1.13 (µmol/L), OSI 1.06±0.14.

Exercise group, it was evaluated for SOD 106.16±13.11 (U/mL), CAT 10.84±2.25 (ng/mL), MDA 22.15±5.56 (nmol/L), GSH 14.35±3.94 (µg/mL), CoQ10 4.02±1.37 (ng/mL), ALA 5.03±0.06 (µg/mL), TAS 1.16±0.40 (mmol/L), TOS 22.07±12.12 (µmol/L), OSI 1.86±0.82.

Lupeol group, it was evaluated for SOD 93.68±22.10 (U/mL), CAT 10.37±3.37 (ng/mL), MDA 51.06±32.71 (nmol/L), GSH 13.71±6.45 (µg/mL), CoQ10 2.80±0.39 (ng/mL), ALA 4.94±0.15 (µg/mL), TAS 0.99±0.43 (mmol/L), TOS 30.11±23.29 (µmol/L), 2.68±1.18 for OSI.

Exercise+Lupeol group, it was evaluated for SOD 74.68±17.76 (U/mL), CAT 16.37±10.06 (ng/mL), MDA 12.52±4.82 (nmol/L), GSH 17.33±12.28 (µg/mL), CoQ10 2.53±0.16 (ng/mL), ALA 4.86±0.08 (µg/mL), TAS 0.62±0.18 (mmol/L), TOS 10.02±5.84 (µmol/L), OSI 1.55±0.55.

As a result of the statistical comparion, there is a significant difference between groups (**Control^a, Exercise^b, Lupeol^c, and Exercise+Lupeol^d)** in SOD ($p<0.05$), MDA ($p<0.01$), CoQ10 ($p<0,01$), ALA ($p<0,05$). TAS ($p<0.05$), TOS) ($p<0.05$), and OSI ($p<0.05$).

When Table 1 is examined, significant differences were found **between Control group^a and Exercise group^b in** the parameters of SOD ($p<0.05$), MDA ($p<0.05$) TAS($p<0.05$), TOS ($p<0.05$), and OSI ($p<0.05$).
Moreover there was signigicant differences between **Control group^a and Lupeol group^c.** The differences were found in MDA ($p<0.01$), CoQ10 ($p<0.05$), TOS ($p<0.05$), and OSI ($p<0.05$).

It is shown in Table 1 that there is signigicant difference between **Control group^a and Exercise+Lupeol group^d** for the parameters of CoQ10 ($p<0,01$), ALA ($p<0.01$) and OSI ($p<0.05$).
On the other hand in the comparison of **Exercise group^b and Lupeol group^c,** only a significant difference was found in CoQ10 ($p<0.05$).

In the comparison of **Exercise group^b and Exercise+Lupeol group^d**, there was found a significant difference for SOD ($p<0.05$) and MDA ($p<0.05$), CoQ10 ($p<0.01$), ALA ($p<0.05$), TAS ($p<0.05$), and TOS ($p<0.05$) values.

Last but not least a significant difference was found between **Lupeol group^c and Exercise+Lupeol group^d** in MDA ($p<0.01$).

Table 1. Differences between groups according to some parameters.

	Group	Min.	Max.	Mean	St. Deviation	Mean of Rows	p	Differences Between Groups
SOD	Control[a]	77.60	107.10	85.55	10.81	9.83	**.046***	**a-b p<0.05**
	Exercise[b]	86.60	122.50	106.16	13.11	18.50		**b-d p<0.05**
	Lupeol[c]	63.90	125.00	93.68	22.10	13.83		
	Exercise+Lupeol[d]	47.70	89.50	74.68	17.76	7.83		
CAT	Control[a]	10.45	15.28	11.78	1.97	14.33	.500	
	Exercise[b]	8.45	14.09	10.84	2.25	11.67		
	Lupeol[c]	8.00	16.98	10.37	3.37	9.25		
	Exercise+Lupeol[d]	7.77	32.81	16.37	10.06	14.75		
MDA	Control[a]	6.67	21.46	13.03	5.00	7.17	**.001****	**a-b p<0.05**
	Exercise[b]	14.95	30.04	22.15	5.56	15.50		**a-c p<0.01**
	Lupeol[c]	22.17	98.71	51.06	32.71	20.50		**b-d p<0.05**
	Exercise+Lupeol[d]	6.65	18.22	12.52	4.82	6.83		**c-d p<0.01**
GSH	Control[a]	1.12	41.43	13.23	15.87	9.33	.646	
	Exercise[b]	9.83	18.81	14.35	3.94	13.83		
	Lupeol[c]	6.77	21.76	13.71	6.45	13.00		
	Exercise+Lupeol[d]	4.96	37.78	17.33	12.28	13.83		
CoQ10	Control[a]	3.01	7.21	4.39	1.84	17.92	**.005****	**a-c p<0.05**
	Exercise[b]	2.66	6.40	4.02	1.37	17.17		**a-d p<0.01**
	Lupeol[c]	2.32	3.32	2.80	0.39	9.25		**b-c p<0.05**
	Exercise+Lupeol[d]	2.36	2.77	2.53	0.16	5.67		**b-d p<0.01**
ALA	Control[a]	4.96	5.02	4.98	0.02	13.92	**.022***	
	Exercise[b]	4.91	5.10	5.03	0.06	18.25		**a-d p<0.01**
	Lupeol[c]	4.73	5.14	4.94	0.15	12.00		**b-d p<0.05**
	Exercise+Lupeol[d]	4.75	4.95	4.86	0.08	5.83		**c-d p<0.01**
TAS	Control[a]	0.59	0.84	0.73	0.10	10.25	**.037***	
	Exercise[b]	0.73	1.87	1.16	0.40	18.58		**a-b p<0.05**
	Lupeol[c]	0.64	1.65	0.99	0.43	13.83		**b-d p<0.05**
	Exercise+Lupeol[d]	0.38	0.82	0.62	0.18	7.33		
TOS	Control[a]	6.28	9.06	7.75	1.13	6.83	**.016***	**a-b p<0.05**
	Exercise[b]	8.52	41.50	22.07	12.12	17.00		**a-c p<0.05**
	Lupeol[c]	6.83	60.07	30.11	23.29	17.17		**b-d p<0.05**
	Exercise+Lupeol[d]	4.74	21.40	10.02	5.84	9.00		
OSI	Control[a]	0.79	1.23	1.06	0.14	5.75	**.033***	**a-b p<0.05**
	Exercise[b]	1.09	3.14	1.86	0.82	14.00		**a-c p<0.05**
	Lupeol[c]	0.96	4.06	2.68	1.18	17.50		**a-d p<0.05**
	Exercise+Lupeol[d]	1.05	2.61	1.55	0.55	12.75		

* = p<0.05, ** = p<0.01

3. DISCUSSION

Acute exercise, especially when done at high intensity, leads to an increase in free radical production due to increased oxygen consumption. Lupeol is known for its antioxidant properties, as well as its free radical inhibitory properties in neuroinflammatory, anticancer and anti-inflammatory conditions [10, 15].

With this research, the protective effects of lupeol on acute exhaustive exercise-related oxidative stress in male rats were investigated.

In our research, statistical differences were found in MDA, CoQ10 between control, exercise, lupeol and exercise+lupeol groups. Significant differences were found in SOD, ALA, TAS, TOS, OSI parameters. There were no significant differences in CAT and GSH. Depending on these values, lupeol has been revealed to play an important role in suppressing free radicals formed in severe acute exercise as well as antioxidant. Lupeol is thought to support antioxidant capacity in the pursuit of severe exercise with its inflammation relieving feature in relation to its triterpenic structure in high-severe exercise. Lupeol is a triterpene and is expected to work just like steroid hormones in high-severe acute exercises, reducing inflammation, and prolonging the anabolic process thanks to its analgesic effect [16]. In our research, the fact that SOD, CAT, MDA are in favor of the lupeol+exercise group, proved that lupeol positively affects the antioxidant system during exercise.

Studies on humans are less compared to studies on experimental animals. However, the effects of exercise on humans can be studied in skeletal muscle tissue and blood. Zergeroğlu et al. [17] performed 30 minutes of exercise in bicycle ergometer 3 times a week for 6 weeks for sedentary people at 75% of the maximum heart rate. He noted that after the 1st week of loading, SOD activity in erythrocytes did not change and SOD activity in erythrocytes increased significantly after the 3rd and 6th weeks of loading.

Burneikoa et al. [18] showed that there was an increase in serum total antioxidant levels of exercises chronically performed in rats for 2 to 5 days a week for 8 weeks. They concluded that SOD levels increased in liver enzymes and CAT levels decreased.

In a similar study in terms of exercise practice, Ji performed aerobic exercise for 8 weeks on different tissues as a result of aerobic activities and concluded that there was no change in heart muscle antioxidant values [19].

Elosua et al. [20] noted that antioxidant enzyme activity increased after 16 weeks of aerobic exercise in 65-80% of max VO_2. It has been reported that SOD activity in erythrocytes was significantly higher than pre-training values after 30, 60, 120 minutes and 24 hours after the 30-minute test performed at the end of the 16-week training program.

In our research, there was a significant difference between SOD, MDA, TAS, TOS, OSI and values between control and exercise groups. There were no significant differences in other parameters.

Physical exercise also causes some changes in enzymatic antioxidant activity or concentrations of non-enzymatic antioxidants. Many studies have shown increased SOD, GSH and CAT activity in tissues or blood after aerobic exercise in both humans and animals [19, 21, 22]. In this study, we can evaluate that CAT and GSH are similar to the results of our research and that they do not show similarity in the SOD enzyme. In this case, the severity of the activity in the acute exercise applied in the research varies according to the energy system spent, and the need for high amounts of oxygen in the anaerobic system, that is, the organism, in the intracellular antioxidant enzymes. As a result, it was concluded that the literature knowledge parallels the results of the study of oxidative stress and free radical formation. Jamurtas et al. [23] compared long and short distance runners based on the idea that different types of exercises have different effects on the antioxidant defense system. They reported that CAT activity was three times higher in long-distance runners than short-distance runners. They reported that CAT activity in long-distance runners is associated with maximum oxygen uptake. Zergeroğlu et al. [17] examined the effect of chronic aerobic exercise on the antioxidant defense system in 14 sedentary men between the ages of 18 and 21. They performed measurement and tests with a load of 30 minutes up to a maximum of 75% of the heart rate max to the bicycle ergometer three times a week for 6 weeks. The activity of SOD and CAT enzymes in erythrocytes and the level of MDA in platelets were measured in blood samples taken before and after exercise, as well as before and after the first exercise. After the first loading, there was no significant difference in SOD and CAT activity in erythrocytes, while at the end of the 3rd and 6th weeks there was a significant increase in SOD activity in erythrocytes after exercise, however, no significant change in CAT activity in erythrocytes and MDA level in platelets was detected. In line with these results, it can be said that endurance training has developed the first stage of the antioxidant defense system. When this study was examined, there was a significant difference in the SOD enzyme in our study, while there was no statistically significant difference in the CAT enzyme. Our research is parallel to the work of Zergeroğlu and the others.

Radak et al. [24] have showed that after 60-70 minutes of acute and strenuous exercise, SOD increased significantly in the base and tibia muscles. It has been noted that SOD activity gradually returned to rest within 1-3 minutes and SOD activity continued to increase even after exercise. They reported that the effect of exercise on SOD may depend on the stimulation threshold and exercise time. In our study, there is an exercise-related statistically significant difference in the SOD enzyme, similar to the work of Radak et al. [24].

In our research, there was a statistically significant difference in the enzyme MDA between the control and lupeol groups. There are statistically significant differences in CoQ10, TOS, OSI. There were no statistically significant differences in other parameters. According to these results, it has been concluded that the lupeol supports intracellular and extracellular antioxidant activity, albeit partially, but during activity, antioxidant and oxidant enzymes play a role in suppressing free radicals. Sahlin et al. found that MDA levels increased during intensive aerobic exercise in athletes. They reported that MDA levels increased significantly after two hours of training in footballers exposed to aerobic exercise [25].

In our research, there was a statistically significant difference in CoQ10 and ALA between control and exercise+lupeol groups. There were no statistical significant differences in other parameters. Antioxidant intake is known to have relatively inhibitory and extracellular harmful radicals from high severe exercise, protecting against balance deterioration, and prohibitive properties against the muscle. Thanks to their

natural structure, antioxidants have been identified as a result of research with certain effects thanks to their reducing properties, which may occur differently from synthetic drugs. Vina et al. emphasized that antioxidants such as vitamins C and E create protection and defense against oxidative stress caused by high-severe exercise in humans and rats [26]. Ohishi et al. [27] examined exercise-induced antioxidant enzyme levels in calcium-fed rats. They evaluated the activity of SOD and CAT enzymes. They reported that while CAT activity decreased after exercise, SOD activity did not change. Koz et al. [28] forced rats to swim for varying periods of time and found that post-exercise MDA levels in various muscle tissues increased significantly in proportion to the exercise time. Similarly, Gül et al. [29] floated the rats for 90 minutes and noted that plasma MDA levels increased significantly after exercise.

In our research, there was a statistically significant difference in CoQ10 between exercise and lupeol groups. There were no statistically significant differences in the others. In the light of these data, CoQ10, which acts as an intermediate with free radicals, is active with harmful radicals and singlet oxygen, preventing lipid peroxidation from starting and the structure of molecules. CoQ10, which also acts in the regeneration of other antioxidants, is the first antioxidant to react when exposed to plasma oxidants [30].

Kwong et al. [31] in a study of CoQ10 supplementation in rats on the antioxidant system, offset the carbonyls and improved plasma aminothiol redox status in skeletal muscle mitochondria. They found that mitochondrial pro-oxidant production and antioxidant defense enzymes reduce oxidative stress.

Eriksson et al. [32] revealed that plasma enzyme concentration increased 3 times more in the free radical environment they made on humans, while there were no significant metabolic changes.

Crane [33] reported that the control of intracellular hydrogen peroxide balance and cell membrane transition channels contributed positively to the functionality of the cell signal in redox control, thanks to CoQ10. When the literature was examined, it was emphasized that he had opposing views about CoQ10 and that there were no significant differences in supporting antioxidant capacity. In particular, this was stated to be commercially prepared and caused by its production in different ways. CoQ10 is used in a free radical environment, considering that it responds optimally to general oxidative damage and energy metabolism problems. In general, in metabolic syndrome disorders, patients with heart failure have been found to have low CoQ10 enzymes in blood and heart muscle than normal values. In professional athletes engaged in high-violent performance sports, CoQ10 has been observed to have low concentration and reductive properties [34].

In our study, a significant statistical difference was found in CoQ10, MDA, ALA, SOD, TAS and TOS values between the exercise and exercise+lupeol groups. There were no statistically significant differences in the others.

Marzatico et al. [35] found that MDA values in sprinters and marathoners increased significantly with a single acute aerobic exercise.

Powers et al. [36] used various types of exercise intensity and duration, as well as various types of muscle fiber, to study exercise-related responses to SOD activity in rats. It has been observed that an increase in the activity of the SOD occurs due to the training time in the Soleus muscle and the high training intensity in the gastrocnemius muscle.

In another study, rats were given a 30-minute swimming exercise and levels of (liver, heart and brain) MDA were detected in tissue samples taken immediately after exercise. In the exercise group, liver and heart MDA levels are significantly higher than the non-exercise group, however, it has been found that brain MDA levels are similar in both groups and there is no significant difference between groups [37].

In our research, statistical significant differences were found in MDA (nmol/L) between lupeol and exercise+lupeol groups. There is no statistically significant difference in the others.

Intense single exercise increases SOD activity in liver [13], skeletal muscle [38], heart [39] and erythrocytes [24] is shown. However, SOD activity is intense physical activity [40] or no reduction in heart tissue and erythrocytes [29] and unchanged [41].

In tissue samples taken 24 hours after the last exercise, it has been reported that SOD, CAT and GSH levels have decreased significantly, while MDA levels have increased [42].

As a result of all studies on animals, it is known that different species differ in their biological-biochemical values obtained as a result of exposure to stress sources with different applications according to their biological formation. When the literature information was compared with the results of our study, in some cases similar results were obtained, while differences were evaluated in some time. This information shows us that we can achieve both similar and opposite results with different stress effects of each organism. Also, it can be thought that consuming foods containing lupeol during periods of exercise may be protective against oxidative stress.

4. CONCLUSION

As a result, lupeol played a significant role in the removal of free radicals via SOD. It has been observed that lupeol has a relative effect on the free radicals that are sent to inhibit catalase and whose form changes after the intracellular catalase and superoxide dismutase turns into H_2O_2. It has been observed that it has a relatively effect on free radicals that have been sent to inhibit catalase and whose form has changed. In malondialdehyde, it has been determined that the damage in the cell membrane is minimized in the group using lupeol in the size of cell damage and lupeol does not have a positive or negative effect in CAT, SOD and MDA, GSH. It has been observed that the elements of antioxidant defense mechanism are effective outside the cell in CoQ10 cell regeneration, ALA, which helps lupeol due to the fact that the exercise applied in the research is anaerobic based. It has been observed that lupeol positively contributes to the level of antioxidants produced by the body and helps in suppressing oxidants, and free radicals from oxidative stress resulting from high severe exercise have been reduced by lupeol. Accordingly, it has been determined that the use of lupeol in exercise in animals exposed to oxidative stress inhibits free radicals consisting of stress, and the hypothesis of the study is supportive.

5. MATERIALS AND METHODS

5.1. Animals

A total of 24 Sprague Dawley male rats three months old, weighing and 200-250 g were used. Animals in the laboratory environment at 12:12 automatic photo period, 23 ^{o}C room temperature, 60% ±5 humidity level and standard rat chow, drinking water (ad libitum) in their feeding. In this study, the breeding, maintenance and application of the animals were carried out at University of Düzce Experimental Animals Application and Research Center on 14.10.2022 until the termination of the experiment. In this process, rats were in no way taken out of the central borders of the animals and were not found in different environments. The ethical approved required for our study was obtained at the meeting of the Local Ethics Committee of the University of Duzce Animal Experiments with the decision no. 19/2/7 at the meeting of 19/02/2019.

5.2. Grouping of Rats, Substances and Doses

In the study, animals were selected by random method and divided into groups in themselves:

Group 1, control group (CG) that did not participate in the exercise and did not apply lupeol

Group 2, acute exercise group (AEG) 25 m/min on TME/MAY brand treadmill specially designed for rats

Group 3, rats using lupeol group (LG) but not participating in exercise

Group 4 was created from rats participating in acute exhaustive exercise and also administered lupeol (ELG).

Lupeol used in experimental studies has been isolated from *Cirsium* species using various chromatographic methods and its structure has been elucidated using various spectroscopic techniques (^{13}C-NMR, ^{1}H-NMR and Mass Spectrometry) [11].

Lupeol, which was obtained pure by the work of Sener et al. [11] was injected with intraperitoneal (*i.p.*) administration in 2% Tween 80 for application to rats. Lupeol was administered to the administered group 30 minutes before exercise by injecting *i.p.* 40 mg/kg under anesthesia by giving anesthetic drugs (ketamine/xylazine 90/10 mg/kg) to rats. After acute exhaustive exercise, blood samples were taken after sacrified by cervical dislocation. After the blood received from animals, ELISA analyzes were performed for the examination of SOD, CAT, MDA, GSH, CoQ10, ALA and oxidative stress levels in serum samples.

5.3. Pre-Application Exercise and Application Exercise Protocol of Experimental Animals

Before being included in the exercise protocol, the rats will be accustomed to running on the treadmill and the recommended training exercise will be applied at a speed of 10 m/min for five days [13]. In our research, the acute exhaustive exercise group (AEG) will run on a treadmill specially designed for rats at a speed of 25 m/min and an incline of 5 degrees until it runs out and activity is terminated [14]. In this way, it is thought that free radicals are formed.

5.4. Antioxidant Activity Studies

All antioxidant activity studies were performed by using commercial kits: CAT (BT-lab, E0869Ra), GSH (Elabscience, E-EL-0026), MDA (Otto Science, Otto1001), SOD (Rel Assay, RLD0123), CoQ10 (BT-lab, E2429Ra), ALA (BT-lab, E0035Ra), TAS (Rel Assay, RL0017), TOS (Rel Assay, RL0024).

5.4.1. Catalase (CAT)

For the level of the catalase, the reaction based on the principle of the catalase breaking down H_2O_2 was used. The relevant reaction was terminated by ammonium molybdate. H_2O_2 in form now reacts with ammonium molybdate to form a yellowish complex. CAT activity was calculated by evaluating the absorbance of the resulting yellowish complex at 405 nm. The results were expressed in (ng/mL).

5.4.2. Glutathione (GSH)

Optical density (OD) was measured spectrophotometrically at a wavelength of 450±2 nm. The GSH concentration in the tested samples was calculated by comparing the OD of the samples with the standard curve. The results were expressed in (µg/mL).

5.4.3. Malondialdehyde (MDA)

The MDA level was determined by a method based on its reaction with thiobarbituric acid TBA (at 90-100 °C). In the TBA test reaction, MDA or MDA-like substances and TBA undergo a reaction that leads to a pink pigment formation with maximum absorption at 532 nm. The reaction was carried out at 90 °C at pH 2-3 for 15 minutes. The sample was mixed with two volumes of cold trichloroacetic acid 10% (a/h) for protein collapse. The precipitate was pelleted by centrifugation and one part of the supernatant was reacted in a boiling water bath for 10 minutes with an equal volume of TBA 0.67% (a/h). After cooling, absorption was measured at 532 nm. The results were expressed in (nmol/L).

5.4.4. Superoxide Dismutase (SOD)

It is to accelerate the dismutation of superoxide dismutase to hydrogen peroxide and molecular oxygen of the toxic radical produced during oxidative energy processes. This method is based on the principle of using xanthine and xanthine oxidase to produce superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazole chloride to form a red formazan dye. Superoxide dismutase activity was then evaluated with the degree of inhibition of this reaction. The results were expressed in (U/mL).

5.4.5. Coenzyme Q10 (CoQ10)

Samples were added to the previously coated plateaus. Then biotinized antigen was added. Antigens in samples compete with biotinized antigen to bind to their specific antibody. The washing process was removed by washing the unbound antigen. Avidin-HRP was added and then incubated. Unconnected avidin-HRP, washing process has been removed. Then TMB Substrate was added and color formation was observed. The reaction was stopped by adding acidic stop solution to the reaction medium and the yellow color formed was measured at 450 nm. The density of the developed color is inversely proportional to the CoQ10 concentration in the sample. The CoQ10 concentration in the sample was then determined by comparing the OD of the samples with the standard curve. The results were expressed in (ng/L).

5.4.6. a-Lipoic Acid (ALA)

After adding samples to pre-coated plateaus, a biotinized antigen was added. Antigens in samples compete with biotinized antigen to bind to their specific antibody. The antigen not connected by washing has been removed by washing. Avidin-HRP was added and then incubated. Unconnected avidin-HRP has been removed by washing. Then TMB Substrate was added and color formation was observed. The reaction was stopped by adding acidic stop solution and the yellow color formed was measured at 450 nm. The density of the resulting color is inversely proportional to the concentration of ALA in the sample. The concentration of ALA in the sample was determined by comparing the OD of the samples with the standard curve. The results were expressed in (µg/mL).

5.4.7. Total Antioxidant Status (TAS)

This new automated method is based on the principle of bleaching the characteristic color of a more stable ABTS radical cation (2.2′- Azino-bis(3-ethylbenzothiazolin-6-sulfonic acid)) by antioxidants. Analysis has excellent precision values are lower than 3%. Results are expressed in mmol Trolox equivalent/L.

5.4.8. Total Oxidant Status (TOS)

The basic principle in this new method is that the oxidants in the sample oxidize the iron ion-o-dianicidine complex to the iron ion. In the formation of the oxidation reaction, glycerol molecules in the reaction medium contribute. Iron ion causes the formation of a complex with xylenol and orange in acidic environment. The color density that can be measured spectrophotometrically is proportional to the total amount of oxidant molecules present in the sample. The test solution was calibrated with hydrogen peroxide

and the results were expressed in micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equivalent/L).

5.4.9. Oxidative Stress Index (OSI)

The ratio of TOS to TAS is evaluated as the oxidative stress index (OSI). The TAS unit obtained for the calculation was converted to µmol/L and the OSI value was calculated according to the formula:
OSI= TOS (µmol H_2O_2 equivalent/L)/TAS (µmol Trolox equivalent/L).

5.5. Data Analysis

Statistical analysis of the data was done with SPSS v.22 and Statistica package programs. Mann-Whitney U Analysis was applied in bilateral comparisons and Kruskal-Wallis Analysis in more than two comparisons in the comparison of groups. The significance value of the data is accepted as $p<0.05$.

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