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Protective role of apilarnil against intense exercise-induced liver injury in rats: serological and histopathological evidence

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Abstract

Intense exercise is known to induce oxidative stress and inflammation, potentially leading to liver damage. This study examined the potential protective effects of apilarnil (AP), a natural bioactive compound with antioxidant and anti-inflammatory properties, against exercise-induced liver injury. Thirty-five male *Wistar* albino rats were allocated into five groups: Control, non-exercise (NEX), exercise (EX), EX + AP1 (0.20 g kg^{-1}) and EX + AP2 (0.40 g kg^{-1}). At the end of the 14-day experiment, serum and liver tissue samples were collected for the analysis of histopathological changes, oxidative stress markers, inflammatory cytokines and serum biochemical parameters. Histopathological evaluations revealed substantial liver damage in the EX group. However, in the EX + AP1 and EX + AP2 groups, the severity of these lesions was significantly attenuated. Biochemical analyses demonstrated elevated levels of tumor necrosis factor-alpha, interleukin-1 beta and interleukin-6 in the NEX group were markedly reduced by AP supplementation. Similarly, malondialdehyde levels were increased, while the activities of antioxidant enzymes - catalase, superoxide dismutase and glutathione peroxidase were declined in the NEX group. AP supplementation reversed these effects by lowering malondialdehyde levels and enhancing antioxidant enzyme activities in the EX + AP1 and EX + AP2 groups. Additionally, serum biochemical analyses indicated improved lipid profiles and liver function parameters in the AP -treated groups compared to the NEX group. In conclusion, histopathological and biochemical findings indicated that AP supplementation mitigated exercise-induced liver damage by reducing oxidative stress and inflammation, while enhancing antioxidant defenses.

Introduction

Intense and exhaustive exercise-induced tissue damage can lead to serious health issues for individuals engaged in heavy sports and physical activities. These types of exercises can cause cell and tissue damage by increasing oxidative stress and triggering inflammatory responses in the body.[1] Oxidative stress develops as a result of excessive free radical production and the insufficiency of antioxidant defense mechanisms, potentially leading to various chronic diseases in the long term.[2] The liver, as the body primary detoxification organ, is one of the most affected by the stress caused by such exercises and is particularly vulnerable to damage following intense physical activity.[3]

The evaluation of specific serum biochemical markers is crucial for assessing liver function and the extent of damage. Additionally, histopathological assessment is a key method for identifying hepatic damage.[4] Serum levels of total bilirubin (TB), direct bilirubin (DB), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total protein, albumin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and unsaturated iron-binding capacity (UIBC) are also essential parameters that reflect liver functional status.[5]

Various biomarkers and enzyme activities have been examined to evaluate liver injury after intense and exhaustive exercise. In particular, the levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL) -1β and IL-6 are measured using enzyme-linked immunosorbent assay (ELISA) methods.[6] Additionally, malondialdehyde (MDA) levels and antioxidant enzyme activities including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are also utilized.[7]

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Apilarnil (AP) is a bee product obtained by grinding and filtering male bee larvae. It is a natural substance that has taken significant interest in the field of health due to its biologically active compounds. Known for its antioxidant and anti-inflammatory properties, AP exhibits protective effects particularly in conditions where oxidative stress and inflammation are prominent.[8-10]

Intense and exhaustive exercise is reported to trigger inflammatory processes and oxidative stress in the body, potentially leading to organ damage. The protective effects of natural substances such as AP against various oxidative stress factors have been emerged as an important research topic in the field of alternative and complementary medicine.[11]

In this study, the protective role of AP against exercise-induced liver damage in rats was investigated both serologically and histopathologically and the regulatory role of AP on impaired biochemical and oxidative stress parameters was examined.

Materials and Methods

Animals. In the present study, a total number of 35 male Wistar albino rats, weighing 350 - 380 g and 14 weeks old were used and the rats were divided into five equal groups (n = 7). During the 14-day application period, the rats were housed in an environment with 55.00% relative humidity, a constant temperature of 24.00 - 25.00 ˚C, a 12-hr light-dark cycle and they were fed ad libitum. This study was conducted at the Bingöl University Experimental Research Center with the permission of the Bingöl University Local Ethics Committee on Animal Experiments (Ethic Code: 14/06/2022-03/02).

Chemicals. Lyophilized Apilanil was used in the present study. It was obtained from Harşena Bee Products (Amasya, Türkiye).

Extraction and analytical methodology. The AP sample used in this study was analyzed for its phenolic composition using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Dicle University Laboratories. To determine the phenolic content of AP, we employed a validated LC-MS/MS method following a standard solvent extraction procedure. The analysis was performed using a Shimadzu Nexera (Shimadzu Corp., Kyoto, Japan) ultra-high-performance liquid chromatography system coupled with a Shimadzu LCMS-8040 tandem mass spectrometer operating in both positive and negative ionization modes. A C18 reverse-phase column (Agilent Poroshell 120 EC-C18, 150 mm × 2.10 mm, 2.70 µm) was used for chromatographic separation. The mobile phases consisted of ultrapure water with 5.00 mM ammonium formate + 0.10% formic acid (Sigma-Aldrich, St. Louis, USA) (Phase A) and acetonitrile with 5.00 mM ammonium formate + 0.10% formic acid (Phase B). Gradient elution was applied over 45 min with a flow rate of 0.50 mL per min and an injection volume of 5.00 µL. Data acquisition and processing were conducted using LabSolutions software (Shimadzu Corp.) in multiple reaction monitoring mode, which allowed selective and quantitative detection of each compound.[12]

Preparation and administration of AP suspension. Lyophilized AP was dissolved in distilled water, thoroughly mixed with a vortex and after forming a suspension it was administered orally to rats.[4]

Experimental animals. Rats were divided into five groups (n = 7): Control (CN) group: No application was performed on this group. Non-exercise group (NEX): Rats in this group were given physiological saline (0.50 mL) orally for 14 days. Only at the end of the 14th day, the rats were made to swim in the swimming tank until they were exhausted. Exercise group (EX): Rats in this group were subjected to 30 min of swimming exercise daily for 14 days and were given 0.50 mL of saline orally each day. At the end of the 14th day, the rats were made to swim in the swimming tank until they were exhausted. The EX + AP1 group (0.20 g kg^{-1}): Rats in this group were subjected to 30 min of swimming exercise daily for 14 days and were given a daily oral dose of 200 mg mL^{-1} AP solution.[4] At the end of the 14th day, the rats were made to swim in the swimming tank until they were exhausted. The EX + AP2 group (0.40 g kg^{-1}): Rats in this group were subjected to 30 min of swimming exercise daily for 14 days and were given a daily oral dose of 400 mg mL^{-1} AP solution.[4] At the end of the 14th day, the rats were made to swim in the swimming tank until they were exhausted.

Water adaptation and swimming exercise protocol. Rats underwent a water adaptation phase prior to the experiment. This phase involved placing the animals in shallow water (31.00 ± 1.00 ˚C) for brief sessions conducted daily between 9:00 a.m. and 11:00 a.m. in one week. The purpose of this procedure was to reduce stress while avoiding adaptations associated with physical training. The rats underwent a daily 30-min continuous swimming exercise protocol in a tank, without the use of external weights or additional load. To ensure consistent participation in the activity, animals that stopped swimming or physically interacted by clinging to one another were gently encouraged to resume swimming. This approach was employed to maintain uniform exercise intensity, minimize variability and ensure the reliability of the experimental results.[13]

Exhaustion exercise protocol. Except for the CN, rats in all other groups were subjected to swimming in a tank (50.00 cm deep and 130 cm in diameter) until exhaustion in groups. The sign of exhaustion was determined as the point at which the animal stopped struggling to stay on the water surface and sank to the bottom of the tank, remaining motionless for 10 sec.[14]

Collection of samples. At the end of the experimental study, the laboratory animals were euthanized by

decapitation after being anesthetized with a combination of 10.00 mg kg^{-1} xylazine (Bayer, Leverkusen, Germany) and 100 mg kg^{-1} ketamine (Pfizer, Istanbul, Türkiye). The blood samples were collected in anticoagulant-free tubes for biochemical analyses. The collected blood samples were centrifuged at 3,000 revolutions per minute at +4.00 ˚C for 10 min to separate the serums which were then placed in Eppendorf tubes and the samples were stored in a deep freezer at – 80.00 ˚C until their analyses were conducted. The liver tissues taken from the animals after the application were also stored in a deep freezer at – 80.00 ˚C until the analysis was conducted.

Biochemical analysis. Serum levels of TB, DB, GGT, ALP, total protein, albumin, total cholesterol, LDL, HDL, and UIBC were analyzed using standard enzymatic colorimetric and spectrophotometric methods. In liver tissues, MDA levels, GPx, SOD and CAT enzyme activities were measured using spectrometric methods.[15] Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 levels in liver tissue were determined by the Sandwich ELISA method using commercial kits (Shanghai Korain Biotech Co., Ltd., Jiaxing, China).

Processing of tissue samples and histopathological examination. During the necropsy, liver tissues from all animals were promptly resected and the right anterior and posterior lobes of the liver were subjected to routine histopathological examination to provide a sample. For this purpose, liver tissues were transferred to a 10.00 % neutral buffered formalin solution and fixed for 48 hr. After the fixation process, the tissues were dehydrated with ethanol solutions of increasing concentrations (50.00, 70.00, 80.00, 96.00, and 100%), cleared with xylene and then subjected to the paraffinization process to prepare paraffin blocks. Four µm thick sections were taken from each paraffinized tissue block using a rotary microtome (2125RT; Leica Nussloch, Germany) and transferred to slides. Slides containing paraffin-embedded tissue samples were stained with Hematoxylin-Eosin after deparaffinization and rehydration processes.[16] Finally, the slides were examined using a light microscope (DM2500 & DFC295; Leica,) adapted for imaging systems.

Histopathological evaluation. Histopathological lesions in liver tissues were semi-quantitatively scored by averaging five different randomly selected and non-overlapping areas at 20 × objective magnification using a light microscope. In liver tissues, sinusoidal dilation and congestion, inflammatory (Kupffer) cell infiltration in the perisinusoidal regions, hydropic degeneration in hepatocytes and Remark cord irregularity were evaluated in accordance with reference studies with a small modification. Accordingly, the presence of Kupffer cell infiltration in the perisinusoidal region was graded as follows: (0: 0.00%, +1: less than 20.00%, +2: 20.00 - 70.00%, 3: more than 70.00% of hepatic lobules), while other parameters were graded from zero to three (0: No lesion; 1: Mild; 2: Moderate; 3: Severe) and scored as the final average of the relevant parameters.[4,17]

Statistical analysis. The study data were analyzed using SPSS Software (version 27.0; IBM Corp., Armonk, USA). The statistical analysis of the histopathological result data was also performed using the Chi-square test. The Kruskal-Wallis test was used to check whether the differences between more than two independent groups were significant for each evaluated lesion. Differences between groups were determined using the Bonferroni test and a $p < 0.05$ was considered significant. The relationship between the groups was calculated using the Pearson correlation coefficient.

Results

Bioactive composition of AP. The analysis showed multiple bioactive phenolic compounds including quinic acid (29.86 mg g^{-1}), fumaric acid (1.57 mg g^{-1}), aconitic acid (0.09 mg g^{-1}), gallic acid (0.32 mg g^{-1}), protocatechuic acid (1.23 mg g^{-1}), catechin (0.40 mg g^{-1}), gentisic acid (1.51 mg g^{-1}), chlorogenic acid (0.03 mg g^{-1}), protocatechuic aldehyde (0.04 mg g^{-1}), 4-OH benzoic acid (4.40 mg g^{-1}), caffeic acid (3.80 mg g^{-1}), vanillin (0.24 mg g^{-1}), p-coumaric acid (6.08 mg g^{-1}), sinapic acid (0.25 mg g^{-1}), salicylic acid (0.08 mg g^{-1}), cyranoside (0.36 mg g^{-1}), isoquercitrin (0.12 mg g^{-1}), and genistin (0.07 mg g^{-1}) among others. These compounds are known for their antioxidant and anti-inflammatory properties which may contribute to the observed protective effects.

Histopathological evaluation. The liver tissues of the animals in the CN had a normal histological structure and no significant histopathological changes were observed. In the NEX group, only a limited level of sinusoidal dilation and congestion as well as hydropic degeneration in hepatocytes were observed, while the liver tissues of the animals in this group generally exhibited a normal histo-morphological structure. The relevant histopathological lesions were most prominently detected in the EX-group and it was determined that the severity of these lesions ranged from mild (+1) to moderate (+2). While there was a partial reduction in the severity of histopathological lesions in the EX + AP1 group, a significant reduction in the presence and scores of histopathological lesions was observed in the EX + AP2 group ($p < 0.05$). It was noteworthy that the most common and prominent lesions observed in the liver tissues of the exercised animals were sinusoidal dilation and congestion, along with hydropic degeneration in the hepatocytes. The results related to the histopathological lesions detected in liver tissues and the histopathological lesion scores for these lesions are specified in Figure 1 and Table 1.

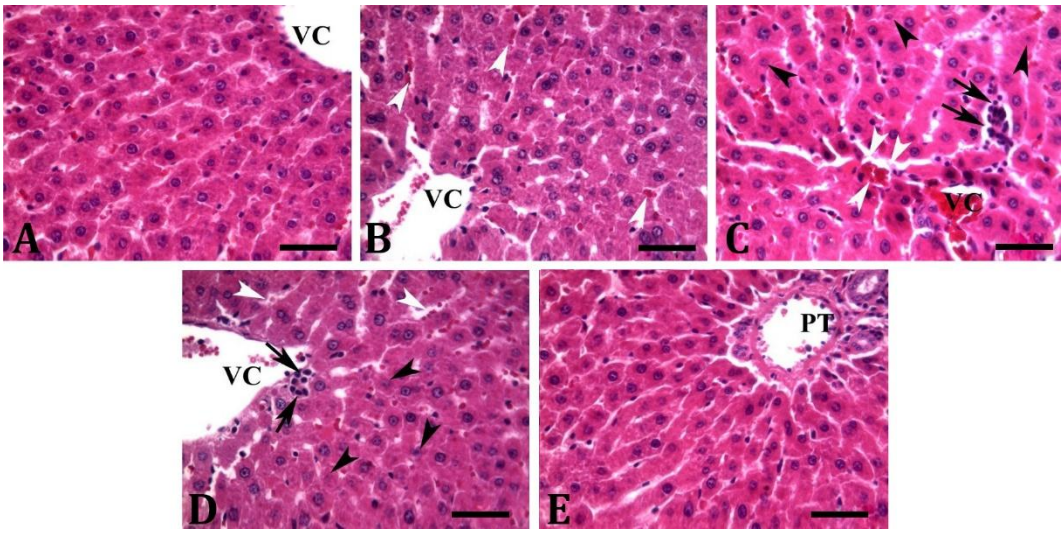

Fig. 1. Microscopic view of liver tissues. **A)** Control group normal histologic appearance. **B)** Non-exercise group. **C)** Exercise group, moderate (+2) changes. **D)** Exercise and apilarnil1 group (0.20 g kg^{-1}), mild (+1) changes. **E)** Exercise and apilarnil2 group (0.40 g kg^{-1}). Black arrowheads: Hydropic degenerated hepatocytes with pyknotic nuclei. White arrowheads: Sinusoidal dilatation and congestion. Black arrows: Increased inflammatory (Kupffer) cell infiltration in the periacinar region. VC: Vena centralis, PT: Portal triad. (Hematoxylin and Eosin staining; Bars = 50.00 µm).

Table 1. Histopathological lesion score statistics of liver

Parameters	CN	NEX	EX	EX + AP1	EX + AP2	*p-value*
Sinusoidal dilatation and congestion	0.03^{a}	0.15^{a}	1.75^{b}	0.80^{c}	0.20^{a}	< 0.05
Inflammatory cell infiltration	0.00^{a}	0.00^{a}	1.06^{b}	0.51^{c}	0.08^{a}	< 0.05
Hepatocyte degeneration	0.03^{a}	0.09^{a}	1.37^{b}	0.63^{c}	0.11^{a}	< 0.05
Remark cord irregularity	0.00^{a}	0.00^{a}	1.11^{b}	0.49^{c}	0.06^{a}	< 0.05

NEX: Non-exercise; EX: Exercise; AP: Apilarnil

^{abc} Columns with different letters indicate statistical differences compared to the control (CN) group, determined by Bonferroni multiple comparison test $p < 0.05$.

Serum biochemical evaluation. In the NEX group, TB, DB, GGT and ALP levels were found to be significantly higher than the other groups (Table 2). There was a significant decrease in TB levels in the AP-supplemented groups. There was a significant decrease in DB, GGT and ALP levels in EX, EX + AP1 and EX + AP2 groups compared to NEX group. The HDL levels were significantly higher in EX, EX + AP1 and EX + AP2 groups compared to NEX group. The LDL and UIBC levels were significantly lower in EX, EX + AP1 and EX + AP2 groups compared to NEX group (Table 2). Albumin levels were higher in EX + AP2 group compared to NEX and EX groups ($p < 0.05$).

Tissue ELISA evaluation. It was observed that TNF-α levels were lower in EX, EX + AP1 and EX + AP2 groups compared to the NEX group. Especially, the decrease in EX + AP2 group was more pronounced and found to be significant compared to NEX and EX groups ($p < 0.05$; Fig. 2A). IL-1β levels were significantly higher in the NEX group compared to the EX, EX + AP1, and EX + AP2 groups ($p < 0.05$; Fig. 2B). The decrease in EX + AP2 group was more pronounced and found to be significant compared to the EX and EX + AP1 groups ($p < 0.05$). Similar results to IL-1β levels were found between the groups ($p < 0.05$; Fig. 2C).

Tissue spectrometric evaluation. In the groups supplemented with AP (EX + AP1 and EX + AP2), the lowest levels of MDA were observed. Compared to the NEX group, these groups exhibited a statistically significant reduction in MDA levels, which was further enhanced with exercise ($p < 0.05$; Fig. 3A). When comparing SOD levels with the CN, NEX and EX groups, higher levels were found in the EX + AP1 and EX + AP2 groups, and this difference was determined to be significant. It was found that SOD levels were significantly increased in the groups supplemented with AP ($p < 0.05$; Fig. 3B). Catalase levels were the lowest in the NEX group, while significant increases were observed in the EX, EX + AP1 and EX + AP2 groups. In the EX + AP2 group, this increase was even more pronounced ($p < 0.05$; Fig. 3C). Glutathione peroxidase levels were also the lowest in the NEX group, and a significant increase was observed in the EX, EX + AP1 and EX + AP2 groups. In the EX + AP2 group, this increase became even more pronounced and significant differences between the groups were identified ($p < 0.05$; Fig. 3D).

The relationship between TNF-α, IL-1β, and IL-6 levels. Analysis of liver cytokine levels revealed strong positive correlations between TNF-α, IL-1β and IL-6 levels.

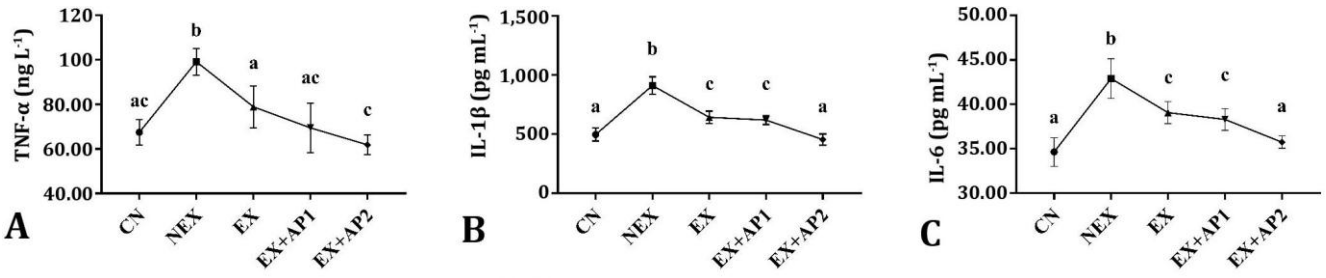

Fig. 2. Enzyme-linked immunosorbent assay results of liver tissue. **A)** Liver tissue tumor necrosis factor-alpha (TNF-α), **B)** interleukin (IL)-1β and **C)** IL-6 levels. NEX: Non-exercise; EX: Exercise; AP: Apilarnil.
[abc] Columns with different letters indicate statistical differences compared to the control (CN) group determined by Bonferroni multiple comparison test $p < 0.05$.

Table 2. Biochemical parameters of serum.

Parameters	CN	NEX	EX	EX + AP1	EX + AP2	*p-value*
TB (mg dL^{-1})	0.05 ± 0.02^{a}	0.07 ± 0.02^{b}	0.06 ± 0.02^{ab}	0.03 ± 0.01^{ac}	0.03 ± 0.01^{ac}	0.0119
DB (mg dL^{-1})	0.04 ± 0.00^{a}	0.06 ± 0.02^{b}	0.03 ± 0.02^{a}	0.02 ± 0.01^{a}	0.01 ± 0.01^{a}	<0.0001
GGT (U L^{-1})	2.79 ± 0.64^{a}	4.20 ± 0.49^{b}	3.27 ± 0.60^{a}	3.16 ± 0.53^{a}	3.01 ± 0.46^{a}	0.0035
ALP (U L^{-1})	246.10 ± 85.55^{a}	445.30 ± 117.30^{b}	293.10 ± 71.20^{a}	251.00 ± 35.78^{a}	287.00 ± 55.69^{a}	0.0006
TP (g dL^{-1})	64.70 ± 3.52	67.73 ± 3.79	64.31 ± 2.99	66.60 ± 1.63	67.14 ± 1.55	0.0572
ALB (g dL^{-1})	33.60 ± 1.29^{a}	33.40 ± 1.34^{a}	35.03 ± 1.17^{b}	35.53 ± 1.32^{ab}	36.99 ± 2.13^{b}	0.0008
TC (mg dL^{-1})	44.76 ± 6.04^{a}	52.60 ± 5.06^{ab}	53.32 ± 2.23^{b}	54.17 ± 6.42^{b}	55.39 ± 4.46^{b}	0.0039
LDL (mg dL^{-1})	10.32 ± 0.97^{ac}	12.67 ± 1.46^{b}	9.83 ± 0.33^{c}	10.39 ± 0.97^{c}	10.47 ± 0.66^{c}	0.0016
HDL (mg dL^{-1})	29.55 ± 4.69^{ac}	26.14 ± 6.79^{a}	34.33 ± 3.15^{c}	34.36 ± 1.79^{c}	33.81 ± 2.01^{c}	0.0082
UIBC (µg dL^{-1})	306.60 ± 39.36^{ab}	335.80 ± 74.55^{a}	283.70 ± 39.26^{ab}	270.60 ± 60.56^{ab}	238.40 ± 73.35^{b}	0.0459

NEX: Non-exercise; EX: Exercise; AP: Apilarnil; TB: Total bilirubin; DB: Direct bilirubin; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; TP: Total protein; ALB: Albumin; TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; UIBC: Unsaturated iron-binding capacity.
[ab] Columns with different letters indicate statistical differences compared to the control (CN) group, determined by Bonferroni multiple comparison test $p < 0.05$.

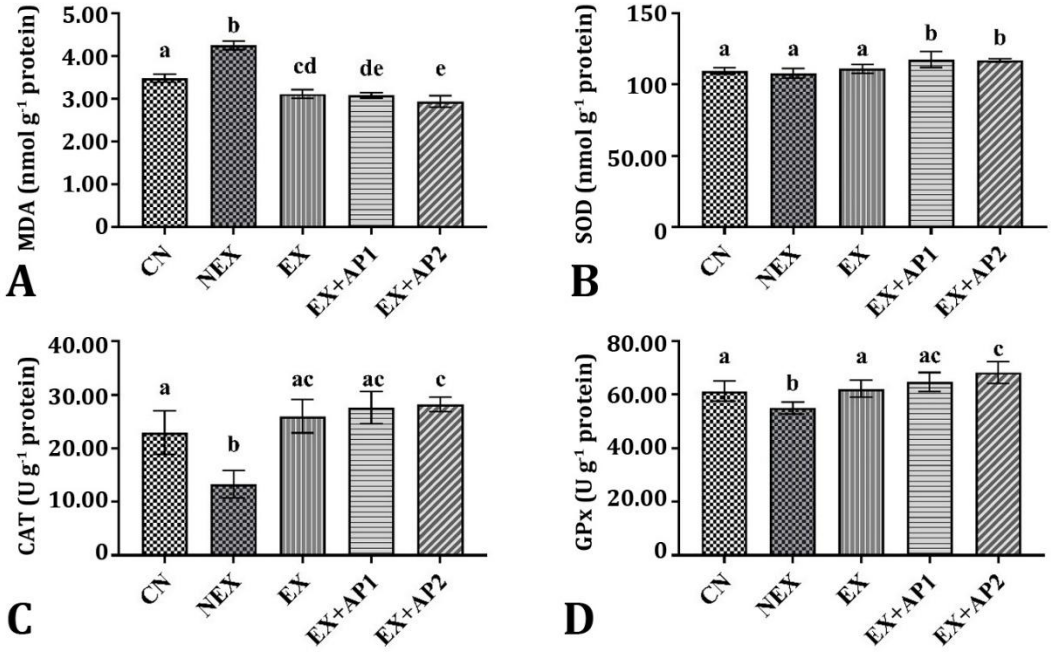

Fig. 3. Spectrometric measurement results of liver tissue. **A)** Malondialdehyde (MDA) level, **B)** superoxide dismutase (SOD), **C)** catalase (CAT), and **D)** glutathione peroxidase (GPx) enzyme activities. NEX: Non-exercise; EX: Exercise; AP: Apilarnil.
[abc] Columns with different letters indicate statistical differences compared to the control (CN) group, determined by Bonferroni multiple comparison test $p < 0.05$.

Statistically significant correlations were found between TNF-α and IL-1β at r = 0.991r, $p < 0.001$, between TNF-α and IL-6 at r = 0.984r, $p < 0.001$ and between IL-1β and IL-6 at r = 0.985r, $p < 0.001$.

Correlation between CAT, SOD and GPx levels. The correlation between CAT, SOD and GPx enzymes in liver tissue was analyzed, and statistically significant strong positive correlations were found. Significant correlations were found between GPx and CAT at r = 0.995, $p < 0.001$, between GPx and SOD at r = 0.993, $p < 0.001$ and between CAT and SOD at r = 0.993, $p < 0.001$.

Discussion

Depending on its duration and intensity, exercise is reported to increase oxidative damage and trigger inflammation.[18] Oxidative stress occurs as a result of increased production of free radicals due to increased oxygen consumption during exercise. These free radicals can damage cellular components leading to lipid peroxidation, protein oxidation and DNA damage.[19] However, it is also reported that acute strenuous exercise increases lipid peroxidation in the liver and causes oxidative damage.[20]

It has been reported that intense exercise increases the release of liver enzymes ALP and GGT and the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6.[21] The duration and intensity of exercise are important factors that determine the degree of these biochemical changes. Prolonged and intense exercise may induce more oxidative stress and inflammatory responses.[3,22] Thus, post-exercise recovery processes and antioxidant defense mechanisms are important to minimize exercise-induced tissue damage. Proper nutrition, rest and the use of supportive supplements during and after exercise can reduce these negative effects.[23]

There has been more research recently on the roles of natural products like AP in these processes. The antioxidant and anti-inflammatory properties of AP offer a potential solution for reducing oxidative stress and inflammatory responses caused by intense exercise. For this reason, it is believed that AP supplementation may have positive effects on the post-exercise recovery process and overall health.[24,25] Although there are studies demonstrating the protective effects of AP on the liver,[4] the results of these studies remain limited. In this regard, we believe that the present study would make a significant contribution to literature in this field.

In our study, considering the biochemical parameters in the serum, it was clearly seen that intense exercise adversely affected liver functions and caused an increase in pro-inflammatory cytokines and enzyme levels that could induce liver damage. The significantly higher levels of TB, DB, GGT and ALP in the NEX group compared to the other groups supported this situation. According to the study results, it is known in the literature that intense exercise leads to an increase in liver enzymes, which is an indicator of liver damage.[26] The elevation of these enzymes indicates liver dysfunctions such as hepatocellular damage.[27]

In the groups supplemented with AP (EX + AP1 and EX + AP2), the significant decrease in TB levels indicated the protective effect of AP against liver dysfunction. The effect of AP was consistent with the hepatoprotective properties of natural products in the literature.[28] The hepato-protective effects of AP could be attributed to the clearance of free radicals and the strengthening of antioxidant defense systems due to the flavonoids and phenolic compounds it contains.[29]

In all groups where exercise and AP application were performed, significant decreases were observed in DB, GGT and ALP levels compared to the NEX group. This situation suggested that AP supplementation, when combined with intense exercise, might improve liver functions. The decreases in GGT and ALP levels in the AP groups indicated that AP increased the liver detoxification capacity.[30]

In terms of HDL levels, the significantly higher levels in the EX, EX + AP1 and EX + AP2 groups compared to the NEX group supported the positive effects of AP on cardiovascular health when combined with exercise. HDL carries cholesterol to the liver, helps clear it and reduce cardiovascular risk factors.[31] Similarly, decreases in LDL and UIBC levels have also been observed in these groups. The decrease in LDL cholesterol reduces the risk of cardiovascular disease and the decreases in UIBC levels indicate positive changes in iron metabolism.[32] High LDL levels can lead to fat accumulation in the liver increasing the risk of fatty liver disease and low UIBC levels may indicate liver dysfunction, and iron accumulation can damage liver tissue.[33]

Microtraumas occurring in muscle tissue during exercise and the resulting inflammatory response can lead to an increase in inflammatory cytokines.[34] The increase in inflammatory cytokines such as TNF-α, IL-1β, and IL-6 may contribute to the development of chronic inflammation and related diseases.[35] In the literature, it has been reported that natural antioxidants and anti-inflammatory agents can reduce inflammation, thus, help prevent chronic diseases.[36] It is reported that flavonoids and phenolic compounds contained in AP can provide these effects by inhibiting inflammatory pathways.[37] An examination of the TNF-α, IL-1β and IL-6 levels of the NEX group revealed that these values were significantly higher in the NEX group compared to the other groups. This result indicated that intense exercise increased the inflammatory response. Significant decreases in TNF-α, IL-1β and IL-6 levels were observed in the AP supplementation and EX groups (EX, EX + AP1 and EX + AP2). These decreases were more pronounced especially

in the EX + AP2 group. This finding suggested that AP might have had anti-inflammatory effects by suppressing the inflammatory response.

The strong positive correlations between the levels of TNF-α, IL-1β and IL-6 in liver tissue indicated that these cytokines played a joint role in regulating the inflammatory response. The mutual promotion of TNF-α and IL-1β expression contributes to the continuity of the inflammatory response, especially in chronic liver diseases.[38] The increase in TNF-α and IL-1β also triggers the expression of IL-6 leading to a broader cellular response to inflammation which increases the risk of fibrosis and cirrhosis as the disease progresses.[38,39] These results suggest that TNF-α, IL-1β, and IL-6 could be used as potential biomarkers in liver diseases, particularly, targeting TNF-α and IL-1β may slow down disease progression by controlling inflammatory processes.[40]

The MDA levels, used as an indicator of oxidative stress, were found to be the highest in the NEX group, while low levels were observed in the EX + AP1 and EX + AP2 groups. This result indicated that intense exercise leds to oxidative stress, while AP supplementation might reduce this stress. Oxidative stress is associated with the increase of free radicals that can cause cellular damage and chronic diseases.[41] The MDA, a product of lipid peroxidation indicates that cell membranes have been damaged.[42]

The increase in SOD, CAT, and GPx levels in the groups supplemented with AP indicated that AP strengthened the antioxidant defense mechanisms. These enzymes provide cellular protection against oxidative stress preventing cell damage.[43] Superoxide dismutase protects cells from oxidative damage by converting superoxide radicals into hydrogen peroxide.[15] Catalase prevents cellular damage by breaking down hydrogen peroxides into water and oxygen. Glutathione peroxidase detoxifies lipid peroxides and hydrogen peroxides using glutathione.[7]

The strong positive correlations between CAT, SOD, and GPx enzymes in the study indicated that these enzymes worked together to combat oxidative stress in the liver. The SOD enzyme forms the first line of defence by converting superoxide radicals into hydrogen peroxide, a less toxic molecule, subsequently, hydrogen peroxide is converted into water by CAT and GPx. This hierarchical structure reveals that the activity of these enzymes is of critical importance especially in chronic liver diseases.[44] The high correlation between GPx and CAT emphasizes the important role these two enzymes play in neutralizing hydrogen peroxide. Decreases in GPx and CAT activity can lead to the accumulation of reactive oxygen species increasing cellular damage and inflammation.[45] Similarly, changes in the activity of SOD can lead to the accumulation of superoxide radicals and an increase in oxidative damage.[46] These results demonstrated the combined effectiveness of CAT, SOD and GPx in maintaining liver health and suggested that supporting the antioxidant defense system might be important for the prevention of liver diseases.

It is known that exercise can cause hepatocyte damage due to oxidative stress on the liver and similarly, it has been reported in some studies that intense exercise causes oxidative stress in liver tissue.[47] It is particularly noted in the literature that AP shows anti-inflammatory and antioxidant effects in liver tissue.[48] In our study, when the hepatoprotective efficacy of AP supplementation against intense exercise-induced liver injury in rats was examined histopathologically, minimal results were observed in the CN and NEX groups, whereas, significant pathological changes such as sinusoidal dilatation, congestion, inflammatory cell infiltration and hepatocyte degeneration were observed in the EX group. In the EX + AP1 and EX + AP2 groups where AP supplementation was administered, the histopathological lesion scores were lower compared to the EX group, supporting the anti-inflammatory and cell-protective effects of AP.[49] In this study, the lower inflammatory and degenerative changes observed in the EX + AP1, and EX + AP2 groups suggested that the protective effect of AP was increased in a dose-dependent manner. Histopathologically, the sinusoidal dilation and congestion, Kupffer cell infiltration in the perisinusoidal region, hydropic degeneration in hepatocytes, and Remark cord irregularities were observed in liver tissues, along with the increase in levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as other enzyme and oxidative stress parameters. This suggested that intense and exhaustive exercise might indirectly have led to hepatic damage. The LC-MS/MS analysis of AP identified potent antioxidant phenolic compounds, including caffeic acid, chlorogenic acid, protocatechuic acid, gentisic acid, vanillin, p-coumaric acid, and sinapic acid. These compounds are known to neutralize reactive oxygen species, reduce pro-inflammatory cytokine release, and exhibit hepatoprotective effects.[4]

The lack of alignment between histopathological findings and biochemical analyses in our study might stem from the distinct parameters these methods assess. Histopathology primarily reflects localized structural and cellular damage such as hepatocyte degeneration and inflammatory infiltration, which may develop progressively or in specific tissue regions. In contrast, biochemical markers, including cytokines and oxidative stress enzymes provide a systemic overview that may not immediately correspond to histological changes.[50]

Several factors might explain the greater histopathological damage observed in the EX group compared to the NEX group. First, the 14-day exercise regimen in the EX group might have resulted in cumulative oxidative stress and inflammation with persistently elevated reactive oxygen species and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) progressively exacerbating tissue damage over time. Second, prolonged exercise might have depleted

antioxidant defense systems (SOD, CAT and GPx), reducing the liver capacity to counteract oxidative damage and making it more vulnerable to severe histopathological alterations after the final exhaustive exercise session. Third, the continuous exercise protocol might have induced hypoxia-reperfusion cycles in hepatic blood flow disrupting oxygenation and impairing recovery mechanisms. Fourth, chronic exercise might have imposed excessive mitochondrial energy demands leading to ATP depletion and an intracellular energy crisis further intensifying cellular stress responses. Finally, since the NEX group had not undergone prior exercise, the liver tissues might have retained a greater regenerative capacity allowing for more efficient recovery despite acute oxidative stress.

Collectively, these factors suggested that the greater histopathological damage in the EX group might result from cumulative stress, exhausted adaptive mechanisms and metabolic overload. This underscores the importance of integrated and multi-level assessments to better understand the complex interplay between molecular and structural liver changes.

The study was conducted on rats. Therefore, it was difficult to directly translate the results to humans. Economic constraints prevented the use of advanced technological analyzers in the study, making it difficult to examine the effects of AP in more detail at the molecular and biochemical levels. Histopathological examinations were performed on limited tissue samples which limited the capacity to represent the whole liver. Furthermore, the serologic parameters used included only specific biomarkers, limiting the ability to assess liver injury more comprehensively.

This study comprehensively evaluated the effects of AP supplementation combined with exercise on liver functions, inflammatory response and oxidative stress. The results suggested that AP had potential benefits in reducing liver damage and improving overall health. The anti-inflammatory and antioxidant properties of AP were observed to be consistent with the positive effects of natural products on health as reported in the literature. This study was considered important as it could shed light on various researches that will enable more comprehensive application dosages of AP in the future and a detailed understanding of the fundamental mechanisms of its effects.

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Conflict of interest

The authors declare no conflicts of interest.

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