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Interactive Effect of the *Linum Usitatissimum* Extracts and Exercise Rehabilitation on Aorta Endothelial and Heart Tissues Apoptosis Biomarkers

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ABSTRACT

Introduction: As one of the most life-threatening illnesses, cardiovascular diseases are often discerned with a high apoptosis rate because of exposure to the high level of oxidative stresses. The present study has investigated the interaction of the *Linum Usitatissimum* (Lu) and aerobic exercise (Ae) on apoptosis of aortic endothelial and heart tissue in rats intoxicated by H_2O_2.

Methods: 56 male Albino Wistar rats were divided into 7 groups, included HC (Healthy Control), TC (Toxic-Control), Toxic-Lu1 (Received Lu, 5 mg/kg), Toxic-Lu2 (Received Lu, 10 mg/kg), Toxic-Ae (Received Aerobic Exercise), Toxic-Ae+Lu1, and Toxic-Ae+Lu2. Finally, the rats were sacrificed ethically, and the apoptotic biomarkers were measured in isolated aortic endothelial and heart tissues.

Results: The interactive comparisons showed that the Ae and Lu had a significant interactive change on pro-apoptosis biomarkers. The BAX in aortic endothelial (P=0.0011) and heart (P=0.0007), caspase-3 in aortic endothelial (P=0.0006) and heart (P=0.0016), and Bcl-2 in aortic endothelial (P=0.0018) and heart (P=0.0016) have significant interactive changes. No significant independent effect was observed. Post hoc test showed that group Toxic-Ae+Lu2 have the most significant improvement compared to the TC group (P≤0.05).

Conclusions: The simultaneous effect of Ae and Lu supplementation most effectively improved the apoptosis biomarkers and displayed potent cardioprotective effects compared to the singular administration of each intervention. Probably, the short rehabilitation period has caused non-significant independent changes. However, the interaction of Ae and Lu has shortened the treatment period.

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Introduction

Apoptosis is one of the main cellular processes that guarantee homeostasis in organisms, leads to the cells (its poor performance) being condemned to death, and new cells are replaced under extensive control (1). Thus, the apoptosis signaling and effector molecules form a complicated network by which the pro-apoptotic and anti-apoptotic factors lead to balanced apoptosis, not more than the level needed to maintain a normal tissue (2). Pathologic apoptosis occurs when the balanced scale is weighted on one side, meaning apoptosis exceeds or falls short of the standard rate. Hence, disorders in modulating cell death or apoptosis can significantly cause cancer, autoimmune lymphoproliferative syndrome, and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease (3, 4). The Bcl-2 protein family, which has consisted of compliant (puma, Noxa, BAD, BAX) and opposing (Bcl-2, Bcl-x_L, Bcl-w) members of apoptosis, partly regulate this phenomenon (5). Puma and Noxa are the BH3-only protein that inactivates the prosurvival Bcl-2 family proteins and subsequently activates the second pro-apoptotic protein Bax or Bak to induce Cytochrome-C release from the mitochondria outer membrane (MOM) (6). Afterward, Cytochrome-C binds to and stimulates Apaf-1 to form an apoptosome complex, leading to caspases activation. In addition to the p53 gene, the mammalian genome encodes two other transcription-related factors,

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p63, and p73 that appear to stimulate the expression of pro-apoptotic genes (7, 8).

Researches have shown that oxidative stresses, especially H_2O_2, are involved in different diseases by leading normal cells to apoptosis (9). The ROS (Reactive oxygen species) molecules such as H_2O_2 are mainly formed in high-energy demanding tissues like the heart, in which a high level of ATP is produced via oxidative phosphorylation (10). These dangerous molecules could seriously damage cell DNA leading to pro-apoptotic factors and cellular death (11). Many cardiomyopathies are associated with mitochondrial DNA damage, leading to defects in the electron transport chain, and an increase in ROS production and disruption of these essential cellular organelle function and mitochondria as the most crucial organelle for apoptosis regulation and initiation could drive the cell to death in this situation (12, 13). It has been indicated that many medicinal herbs are rich sources of compounds with ant apoptotic effects, which made them worth having pharmaceutical agents (14) so that many studies have shown beneficial influences of various herbs in attenuation of apoptosis in patients with cardiac disorders, which resulted in an improved condition of these people (15).

Flax (*Linum Usitatissimum*) or Flaxseed belongs *Linaceae* plants family, and it is a well-known growing plant in tropical and subtropical regions for general medicinal uses from the past till now (16). It is a pharmaceutically accepted plant because of its high level of beneficial omega-3 fatty acids (PUFA) and other compounds such as lignans, fibers, minerals, and vitamins (17). As reported, the lignans, especially Secoisolariciresinol diglucoside (SDG), potentially possess antioxidant, anti-inflammation, anticoagulant, activity, and cytotoxic effects on some human cancers. Interestingly, *Linum Usitatissimum* has paradoxical activity in apoptosis in cancerous and normal cells, so that researchers have shown that flaxseed extract increases and decreases the apoptosis rate in abnormal and healthy cells, respectively (18). As this plant is a rich source of unsaturated omega-3 fatty acid such as α-linoleic acid (ALA) and regarding ant apoptotic traits of flaxseed, the hypothesis comes up that this plant could benefit cardiovascular disease in 2 ways; it supplies a healthier food with less saturated fatty acids, on the one hand. Secondly, it can diminish the apoptosis severity in damaged cardiovascular tissues (19).

Furthermore, studies have shown that exercise reduces apoptosis by modulation of stress-sensitive proteins such as the nuclear factor NF-κB, insulin-like growth factor (IGF-1), and heat shock protein (HSP90 and HSP70) (20). Furthermore, it is known that aerobic exercise declines the Bax/Bcl-2 ratio and activation of caspases, including 3 and 9, and also fewer DNA fragments are observed in trained rats (21). It is suggested that exercise may stimulate cell survival proteins including MnSO3, MnSOD, NF-kB, extracellular kinase receptor (ERK), IGF-1/Akt pathway, and heat shock protein (HSP) to reduce cell death in the heart (22, 23). Moreover, exercise has shown to be an anti-oxidative intervention as it has been found to elevate anti-oxidative enzymes and free radical scavenger molecules. Thus, many researchers have stated that aerobic exercise could efficiently increase cell survival in patients suffering from cardiovascular disease in which oxidative stress-induced cardiomyocytes death is found (23). As most cardiovascular diseases are accompanied by the high rate of apoptosis in endothelial and cardiomyocyte cells (24), aerobic exercise could be considered a dynamic therapeutic approach.

According to the cardioprotective effects of *Linum Usitatissimum* and exercise, the present study investigates the combined effect of these interventions on the apoptosis rate of heart and aortic endothelial of rats poisoned with H_2O_2 who artificially experienced a high level of apoptosis in heart and aortic endothelial tissues.

Materials & Methods

The Ethical Approval

The ethics committee approved the whole experimental protocol in this study of Islamic Azad University, Mahallat Branch (IR.IAU.ARAK.REC.1399.043). It was done under the NIH (National Institutes of Health) guide for the care and use of laboratory animals (No. 80–23), which emphasized minimal animals being sacrificed and minimal pain imposed during the study.

Animals and Groups

The rats for the present study included 56 Wistar Albino male rats (Ages 10-12 weeks and weight 200 ± 20 g). They were purchased from the Institute of Pasteur, Tehran, Iran. After direct proof of all rat's health, they laid into the 6

separate cages with temperature 22-27 °C and alternative exposure to 12 hours of light and 12 hours of darkness to adapt to the conditions before experiments at the university's Animal Care Facility. The animal also had optional access to water and food in all study periods. Then, the rats were categorized into 7 groups (n=8), and all got poisoned but group HC. The groups included as following: HC (Healthy Control), TC (Toxic-Control), Toxic-Lu1 (Received Lu, 5 mg/kg), Toxic-Lu2 (Received Lu, 10 mg/kg), Toxic-Ae (Received Aerobic Exercise), Toxic-Ae+Lu1, and Toxic-Ae+Lu2.

For treating the rats, TC group was only were poisoned by H_2O_2 without any treatment. Toxic-Lu1 and Toxic-Lu2 Group received 5 and 10 mg/kg of herbal extract, respectively, without aerobic exercise. The Toxic-Ae group experienced only an aerobic exercise program without any herbal extract. Also, Toxic-Ae+Lu1 and Ae+Lu2 groups were delivered 5 and 10 mg/kg of herbal extract, respectively. Besides, they underwent an aerobic exercise program. Eventually, HC group received no poisoning and no treatments. To make sure that no other chemical effect on data, no sweetener substrate was used. The herbal extract was orally administered to rats daily for 28 days (4 weeks) so that in groups 5 and 6, it was fed one hour after every aerobic exercise session in the 2nd and 3rd weeks. To make sure that no other chemical effect on data, no sweetener substrate was used. The herbal extract was orally administered to rats daily for 28 days (4 weeks) so that in groups 5 and 6, it was fed one hour after every aerobic exercise session in the 2nd and 3rd weeks.

H_2O_2 Induced Toxicity

The animals were poisoned using peroxide hydrogen prepared from Atusa oxidants 9% product (Grape Oxidant 6 Number 1 Atusa 60 ml) bought from Atusa Company, Tehran, Iran. This product maintained a lot of hydrogen peroxide (H_2O_2) manufactured for hair dyeing processes. To poison rats, they needed to inhale the oxidants so that 60 mg of oxidant tube was poured inbox with a volume of 125.123 mm^2. The grid-form box did not let oral usage of the substrate by rats. Thus, the rats breathed the air of a cage in which the oxidant-contained box was put. In each poisoning phase, 4-5 mice were allowed to inhale the air for 3 hours a day for one week.

Preparation of Linum Usitatissimum

The seeds (Pakan Bazr Co., Isfahan, Iran) were grinded thoroughly, and 10 g of powder was solved in petroleum ether to prepare Linum Usitatissimum supplementation. The solution was poured into a Soxhlet extractor to extract oil, taking along for 10 hours. In the next step, the solution whose isolated oil was thoroughly dried to purify herbal extract in the next step, in which methanol was added to the dried powder. The primary herbal extract was gained from this mixture using Soxhlet extractorduring16 hours. A yellow solution was obtained as the methanolic extract, kept at 50° C for 5 hours to evaporate the containing methanol. Finally, after drying, the remained yellow powder was dissolved in normal saline and stored a 4 ° C in darkness.

Aerobic Exercise Protocol

The low-intensity interval training (LIIT) was done under 2 separate programs, including adaptation and main. The mice did 4 days of exercise (Each day included 4 times of1 min running at a speed of 20-25 meters per min on a rotational bar). The main program consisted of 1 min of running at 20-25 m/min, followed by 2 min of active rest (running at 10-12 m/min). This protocol was repeated 10 times, meaning that the whole main program took 30 minutes per rat. The main programs were executed 5 sessions a week for 4 weeks (25).

Sacrifice and Laboratory Methods

After the treating period, the cardiovascular tissues were analyzed after 24 hours of the last treating session, during which the rats were not fed for 14h. Qua, the ketamine (30-50 mg/kg), and xylazine (3-5 mg/kg) were utilized via intraperitoneal injection to pass out the rats. After complete anesthesia, a cleft at the center of the rat breast was made using the surgical blade, and the aortic cardiovascular tissue was extracted. Then, the tissues were immediately washed by normal saline and frozen using nitrogen (180 ° C) and stored at -80°C. To be able to assess the apoptotic factors, we first needed to homogenize tissue samples. After the defreeze of tissues, they were transferred into 2 ml microtubes, and 310-340 µl of lysis buffer was added for 62-68 mg of tissue samples, as 500µl of lysis buffer (EPX-99999-000) is recommended for 100 mg of tissue. Significantly, antiprotease existing in lysis buffer prohibited protein

denaturation. We used a 5 mm stainless steel bead in microtubes, which were laid into a TissueLyser device. The Homogenization processes were performed by processing at 25 Hz for 2 minutes and final centrifuge at 4 ° C for 10 minutes. Then, the supernatant was disposed of into new microtubes, and the homogenate sample was diluted at a ratio of 10 mg protein/ml using 1X PBS and was kept at -80 ° C. At the final step, we quantitatively analyzed the pro-apoptotic factors, including Bax (pg/ml) and caspase 3 (ng/ml) (Cas 3) and anti-apoptotic factor Bcl2 (ng/ml) in both heart and aortic endothelial cells using ELISA (Mabtech, Sweden), to assess the influence of *Linum Usitatissimum* methanolic extract and aerobic exercise on cellular apoptosis.

Statistical Analysis

The Kolmogorov-Smirnov test was used to determine the normality of the distribution. All results were expressed as mean ± standard deviation. In order to analyze the data and investigate the inconsistencies of the observation amongst different groups, the two-way analysis of variance ANOVA method was used, followed by the LSD posthoc test. The data were analyzed using the Prism 8 software at a statistically significant level ($P \leq 0.05$).

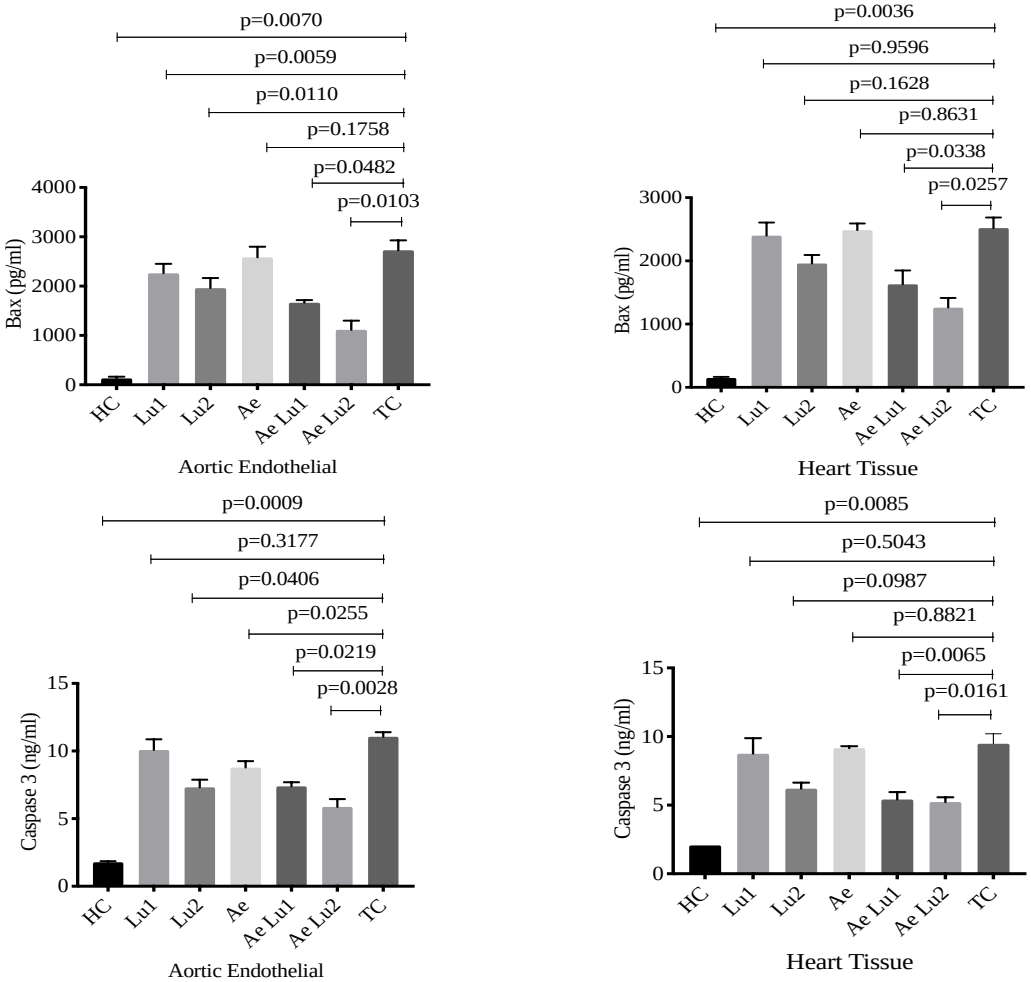

Figure 1. The graphs show the difference between apoptotic biomarkers (Bax and caspase 3) in the studied groups.
*HC (Healthy Control), TC (Toxic-Control), Toxic-Lu1 (Received Lu, 5 mg/kg), Toxic-Lu2 (Received Lu, 10 mg/kg), Toxic-Ae (Received Aerobic Exercise), Toxic-Ae+Lu1, and Toxic-Ae+Lu2.

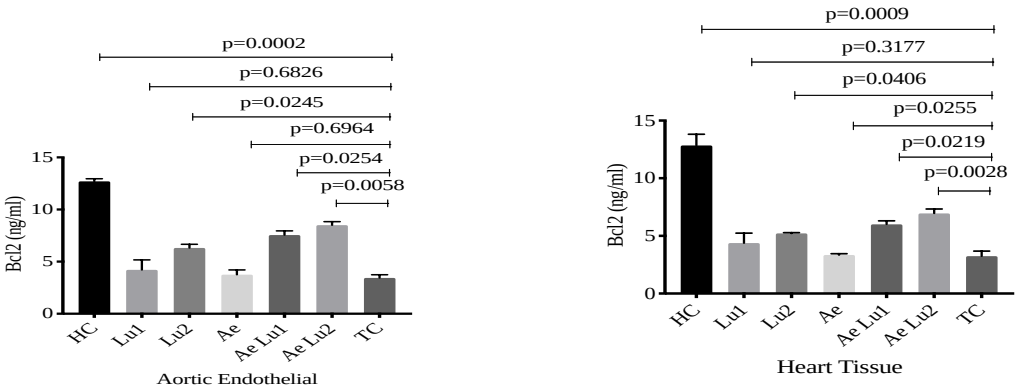

Figure 2. The graphs show the difference of apoptotic biomarkers (Bcl-2) in the studied groups.
*HC (Healthy Control), TC (Toxic-Control), Toxic-Lu1 (Received Lu, 5 mg/kg), Toxic-Lu2 (Received Lu, 10 mg/kg), Toxic-Ae (Received Aerobic Exercise), Toxic-Ae+Lu1, and Toxic-Ae+Lu2.

Results

The Synchronous Administration of Lu and Ae Most Effectively Attenuates Apoptosis Rate in Cardiovascular Tissue

The interactive comparisons showed that the Linum Usitatissimum and aerobic exercise had lessened the pro-apoptosis biomarker BAX in aortic endothelial (F=90.41, p=0.0011, η=0.9784) and heart tissue (F=87.89, p=0.0007, η=0.9777). The same results were found for Cas-3, so that the interventions meaningfully decreased the level of Cas-3 in aortic endothelial (F=114.3, p=0.0006, η=0.9828) and heart tissue (F=71.32, p=0.0016, η=0.9727). No significant independent effect was observed. Post hoc test showed that group Toxic-Ae+Lu2 showed the most significant improvement compared to the TC group for Bax and Caspase-3 (Fig-1). A posthoc test showed a significant difference between the studied groups for Bax in the heart (P=0.0257) and in aortic endothelial (P=0.0103) in the Toxic-Ae+Lu2 group compared to the TC group. A posthoc test showed a significant difference between the studied groups for caspase-3 in the heart (P=0.0161) and aortic endothelial (P=0.0028) in group Toxic-Ae+Lu2 compared to the TC group. Other group comparisons are shown in the figure-1.

Moreover, according to interactive analysis, the Lu and Ae have efficiently decreased the level of antiapoptosis biomarker Bcl2 in aortic endothelial (F=119.3, p=0.0018, η=0.9735) and heart tissue (F=71.32, p=0.0016, η=0. 9805). No significant independent effect was observed. Post hoc test showed that group Toxic-Ae+Lu2 showed the most significant improvement compared to the TC group for Bcl-2 (Fig-2). A posthoc test showed a significant difference between the studied groups for Bcl-2 in the heart (P=0.0028) and aortic endothelial (P=0.0058) in the Toxic-Ae+Lu2 group compared to the TC group. Other group comparisons are shown in the figure-2.

Table 1. The mean level of BAX in studied groups in aortic endothelial and heart tissue cells. The data were analyzed by a two-way ANOVA.

Groups		Mean	F	P.value	η
Aortic endothelial	HC	132.8 ± 31.62	90.41	0.0011	0.9784
	Lu1	2261 ± 191.7			
	Lu2	1962 ± 204.3			
	Ae	2586 ± 212.8			
	Ae Lu1	1667 ± 51.28			
	Ae Lu2	1116 ± 187.3			
	TC	2729 ± 201.1			
Heart Tissue	HC	148.1 ± 17.97	87.89	0.0007	0.9777
	Lu1	2400 ± 206.2			
	Lu2	1958 ± 136.3			
	Ae	2485 ± 105.7			
	Ae Lu1	1630 ± 217.9			
	Ae Lu2	1262 ± 152.6			
	TC	2518 ± 169.6			

Table 2. The mean level of Caspase 3 in studied groups in aortic endothelial and heart tissue cells. The data were analyzed by a two-way ANOVA.

Groups		Mean	F	P.value	η
Aortic endothelial	HC	1.78 ± 0.06	114.3	0.0006	0.9828
	Lu1	10.0 ± 0.77			
	Lu2	7.31 ± 0.56			
	Ae	8.78 ± 0.47			
	Ae Lu1	7.39 ± 0.29			
	Ae Lu2	5.86 ± 0.58			
	TC	11.06 ± 0.33			
Heart Tissue	HC	2.06 ± 0.00	71.32	0.0016	0.9727
	Lu1	8.76 ± 1.12			
	Lu2	6.20 ± 0.44			
	Ae	9.15 ± 0.14			
	Ae Lu1	5.41 ± 0.54			
	Ae Lu2	5.23 ± 0.35			
	TC	9.47 ± 0.72			

Table 3. The mean level of Bcl2in studied groups in aortic endothelial and heart tissue cells. The data were analyzed by a two-way ANOVA.

Groups		Mean	F	P.value	η
Aortic endothelial	HC	3.44 ± 0.29	119.3	0.0018	0.9835
	Lu1	4.21 ± 0.95			
	Lu2	6.32 ± 0.34			
	Ae	3.75 ± 0.44			
	Ae Lu1	7.55 ± 0.41			
	Ae Lu2	8.50 ± 0.23			
	TC	12.7 ± 0.25			
Heart Tissue	HC	3.242 ± 0.2519	71.32	0.0016	0.9805
	Lu1	4.381 ± 0.499			
	Lu2	5.221 ± 0.0342			
	Ae	3.338 ± 0.0714			
	Ae Lu1	6 ± 0.1752			
	Ae Lu2	6.954 ± 0.219			
	TC	12.85 ± 0.5632			

Discussion

The literature strongly supports the increased apoptosis rate in cardiovascular diseases such as ischemia-reperfusion, heart failure, and cardiac hypertrophy (26). The cytochrome C leakage and caspases activation in animals who ischemic heart cells are solid supportive data in this context. Researchers have shown that many cardiovascular disorders are due to oxidative stresses and resulted in ROS molecules, mainly H_2O_2 (27). These hazardous molecules could lead cardiomyocytes to death through various pathways, mainly by macromolecule damage such as DNA and ATP depletion, which put the cells in a stressed condition and initiates apoptosis cascade (28). Thus, many efforts have been made to identify interventions with anti-oxidative effects to attenuate the apoptosis phenomenon in these patients to improve their overall clinical condition and lessen mortality. One of the most studied interventions is a physical exercise that has strongly displayed antioxidant properties. It has been observed that aerobic exercise leads to lessened oxidative-induced apoptosis in animal models with cardiovascular diseases so that the lower level of apoptosis markers such as Bax, Caspase 3, fragmented DNA and Bax to Bcl2 ratio (29).

On the other hand, the studied plant, *Linum Usitatissimum*, has been shown to contain many beneficial chemicals with pharmacologic traits that could benefit patients with cardiovascular diseases partly in 2 ways. *The Lu* is a rich source of many anti-apoptotic compounds include polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA), α-linoleic acid (ALA), and eicosapentaenoic acid (EPA) that play cardioprotective roles (30). Moreover, the anti-apoptotic and anti-inflammation features of Lu have been revealed, while that inflammation and oxidative stresses enhance cardiocyte death in various cardiac disorders (31). Due to reports, it has also been indicated that *Linum Usitatissimum* extract declines the DNA fragmentations, pro-

apoptotic factors caspase 3 and 9, and inflammatory factors such as IL-1, 2, 6, and INF-γ by its containing linoleic acid and SDG compounds (32).

The present study data demonstrated that aerobic exercise and herbal extract might trigger significant alterations in specific apoptosis biomarkers when they were lonely administered to rats. However, the most significant upregulation of Bcl-2 and downregulation of Bax and caspase 3were observed while continuously delivered. In this regard, shirvani et al. implied that the combination of training and flaxseed oil upregulated the expression of cardioprotective engaging genes UCP-2, UCP-3, and eNOS genes more than when each of them was used solitarily (33). These findings indicated that the herbal extract interference could benefit patients with cardiovascular disease because of 2 main characteristics: healthy unsaturated fatty acids (omega-3 fatty acid) and, secondly, due to advantageous cellular activities such as anti-oxidative traits. This study showed, though, the Lu supplementation (at a high dose) or Ae individually may have altered apoptosis biomarkers, but these alterations are intensified in the presence of both interventions. In this regard, Ghosh et al. stated that moderate exercise has caused Bax/Bcl-2 ratio and caspase 3 to be diminished in the hippocampus of rats (34). Following our data, derbali et al. also mentioned that linseed oil has a cardioprotective effect in rats with isoproterenol-induced myocardial infarction as it lowered the level of myocardial infarction, including LDH, ALP, AST, and CK-MB (35). Furthermore, Hasan et al. showed that *Linum Usitatissimum* extracted lessened the troponin I, LDL, VLDL and heightened the level of antioxidant enzymes Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) in rabbits who had experienced the isoproterenol-induced myocardial infarction (36). In support of previous studies, the present study states the robust anti-oxidative features of *Linum Usitatissimum* and LIIT, which could be cardioprotective in cardiovascular tissues.

Conclusions

As our data demonstrated, the Linum Usitatissimum and aerobic exercise could attenuate the apoptosis rate in aortic endothelial and heart tissues. The concurrent administration of both results in more antiapoptosis effects compared to when each of them is applied lonely. Probably, the short training period has caused a non-significant independent changes. However, the interaction of Ae and Lu has shortened the treatment period. Thus, regarding chemicals drug's side-effects and other preventive advantages of natural medications, combining herbal-physical therapy could be a suitable approach in future studies as an efficient treatment approach for cardiovascular diseases.

Conflict of Interest

The authors declare that no conflict of interest exists with the present study.

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