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Original Article

Limonene as a potential cardioprotective agent: mechanistic intuitions into antioxidant and anti-apoptotic properties in isoproterenol-induced ischemia in rats

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Abstract

Background and aims: Excessive oxidative stress and increased apoptosis are the main culprits to heart damage associated with ischemic heart disease. This study aimed to demonstrate the cardioprotective properties of limonene (LIM), a monoterpenoid compound, in isoproterenol-induced ischemia (ISO-I) in rats.

Methods: Forty male Wistar rats were divided into five groups (n=8). Normal saline (1 mL/kg) and LIM at doses of 10 mg/kg, 20 mg/kg, and 40 mg/kg were administered for seven days before the induction of ischemia by ISO. Creatine kinase-MB (CK-MB), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), malondialdehyde (MDA), total antioxidant capability (TAC), and relative expression of *BCL2* and *BAX* genes underwent measurement.

Results: CK-MB (3.73±0.560 versus 0.888±0.317), CPK (901±72.2 versus 397±33.5), LDH (1992±176 versus 821±94.1), and MDA levels in the serum (65.6±11.0 versus 28.3±4.75) and heart (104±8.70 versus 65.3±7.46) samples significantly increased following ischemia in the treatment groups compared to the control group ($P<0.001$ for all markers). Ischemia decreased TAC in the heart (324±45.0 versus 588±64.5) and serum (202±23.0 versus 388±45.9) samples compared to the control counterparts ($P<0.001$ for both samples). ISO-I is associated with a decrease in *Bcl-2* (0.880±0.118 versus 1.74±0.178) and an increase in *BAX* (2.75±0.124 versus 1.08±0.0938) gene expression in comparison to the control group ($P<0.001$ for both genes). LIM could reverse the aforementioned heart damage-related biomarkers in the serum and heart samples. Finally, LIM improved histopathological disarrangement following ischemia.

Conclusion: The findings revealed that LIM, at least partially due to its antioxidant and anti-apoptotic potentials, exerted cardioprotection against ISO-induced heart ischemia in rats.

Keywords: Ischemia, Isoproterenol, Antioxidant, Apoptosis, Limonene

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Introduction

Cardiovascular disease (CVD) accounts for approximately one-third of global mortality. According to an estimation in 2017, nearly 40% of mortality in the American female population was attributed to CVDs, with ischemic heart disease (IHD) being the primary cause of early death (1,2). CVD is significantly associated with high systolic blood pressure, high cholesterol levels, smoking, and diabetes mellitus. These risk factors lead to immune dysregulation and an imbalance between the heart's pro- and anti-atherogenic immune cells. Ultimately, this imbalance leads to postponed clinical manifestations of IHD, such as ischemic cardiomyopathy and necrosis in myocardial muscle, diminishing the heart pump function (3,4).

Recent evidence has emphasized the importance of oxidative stress-mediated inflammation in the early stages of atherosclerotic plaque development, plaque instability, and plaque rupture (5-7). The inflammatory response in the intima causes apoptotic endothelial cell death through reactive oxygen species (ROS) overproduction, nitric oxide synthesis inhibition, and increased oxidized low-density lipoprotein (6,8,9). Lipid peroxidation and degradation of polyunsaturated fatty acids by ROS initiate the process, resulting in inflammation and apoptosis in the heart tissue. Malondialdehyde (MDA) is the end product synthesized during the fat peroxidation cascade, serving as a marker for the severity of coronary disease (10,11).

The B-cell lymphoma/leukemia2 (BCL2) protein family is one of the most well-known pathways that modulates programmed cell death and apoptosis in cardiomyocytes (12). BCL2 antagonist/killer (BAK) and BCL2-associated X protein (BAX) are the primary inducers of apoptosis in cardiomyocytes. On the contrary, the BCL2 protein establishes anti-apoptotic conditions and promotes cell survival. BAX and BAK protein oligomerization in the

mitochondrial outer membrane initiates the response to apoptosis during ischemia. This process eventually releases contents between the inner and outer mitochondrial membranes, prompting the intrinsic apoptosis pathway (12,13). Limonene (LIM) is mainly found in oranges and lemons, indicating anti-inflammatory and anti-apoptotic effects (14). It has been suggested that D- and S- isomers of LIM stimulate anti-tumor and apoptosis induction in malignant cells by suppressing the PI3K/Akt/mTOR17 and NF-κB pathways (15). D-LIM exerts its cardioprotective effects by strengthening the antioxidant capacity and suppressing inflammatory and heart damage mediators (16). LIM may institute cell survival by reversing mitochondrial membrane depolarization changes, reversing mitochondrial ROS level, and inhibiting the NF-κB pathway (17).

Therefore, the current study aims to discover the cardioprotective effects of LIM in an experimental model of cardiac ischemia induced by isoproterenol (ISO) in rats, measuring apoptosis components, heart damage-associated biomarkers, and histopathologic alternations of the heart tissue.

Materials and Methods

Animals

This study was approved by the Ethics Committee of Animal Investigations at Shahrekord University of Medical Sciences (with the ethical code of IR.SKUMS.REC.1398.160). Male Wistar rats (N = 40) weighing 200-250 g were purchased from the Pasteur Laboratory (Pasteur Institute, Tehran, Iran). The animals were kept under standard laboratory conditions (12-hour light/12-hour dark cycle, temperature of 21 ± 5 ˚C, humidity of 50 ± 2, and free access to water and food). The animals were randomly divided into five groups (each containing eight rats), including control + saline treatment (C + ST, 1 mL/kg), ISO-I + saline (1 mL/kg), ISO-I + LIM (ISO-I + LIM10, 10 mg/kg), ISO-I + LIM (ISO-I + LIM20, 20 mg/kg), and ISO-I + LIM (ISO-I + LIM 40, 40 mg/kg).

Limonene administration and ischemia-related injury protocol

ISO hydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA) at 85 mg/kg dissolved in physiological saline was used to induce ischemic injury. LIM was bought from Sigma Company and dissolved in physiological saline to administer at 10 mg/kg, 20 mg/kg, and 40 mg/kg of body weight. The animals were treated with LIM or saline for seven continuous days via the intraperitoneal route. Then, ischemic-related heart injury was induced by the intraperitoneal injection of ISO on two consecutive days with an interval of 24 hours (18). The dosage and duration of treatments were chosen based on our pilot study and previous studies (18,19). After 24 hours from the last dose of ISO, the rats were deeply anesthetized by the intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). After euthanizing, blood samples were taken from the heart, and an incision in the chest was made to separate the roots of the heart vessels and extract the heart for biochemical and histopathological evaluations.

Biochemistry measurements

Blood samples were centrifuged at 3,500 rpm (15 minutes at 4 ˚C) and assessed for creatine kinase-MB (CK-MB), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH) through spectrophotometric absorbance according to commercial kit guidance. MDA, the indicator of lipoperoxidation, was measured using the thiobarbituric acid (TAB) reactive substance method as described previously (20). Concisely, plasma or supernatant was mixed with sodium dodecyl sulfate (8.1%) and TBA/buffer (composed of 0.53% TAB in 20% acetic acid as accustomed to a pH rate of 3.5 with NaOH), then incubated at 95 ˚C for 60 minutes. The reaction was clogged by placing tubes in ice, followed by centrifugation at 4000 rpm for 10 minutes. The optical density of the pink color was estimated at 532 nm. Moreover, 1, 1, 3, 3-tetraethoxypropane was used as a standard of the MDA assay, and the results were expressed in μmol/mg. As previously described (21), the antioxidant capacity was calculated through the ferric reducing antioxidant power (FRAP) method. Total antioxidant capability (TAC) is based on lowering Fe^{+2} and Fe^{+3} ions in the presence of the tris (2-pyridyl)-s-triazine reagent. The reaction of Fe^{+2} with the reagent produced a blue-colored complex with a maximum absorption of 593 nm in spectrophotometry.

Qualitative real-time polymerase chain reaction protocol

After collecting the heart tissues, total RNA was extracted utilizing RNX-plus. The RNA was then reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). Gene-specific primers for *BCL2* and *BAX* genes were designed and optimized, and RT-PCR was performed on the cDNA samples with a light cycler instrument (Roche Diagnostics, Mannheim, Germany; Takara Bio). The outcomes were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression of *BCL2* and *BAX* genes in the heart. The housekeeping gene *B2M* was used as a reference gene to normalize the gene expression levels (22). Table 1 presents primer sequences.

Histopathological analysis

After washing with a solution containing potassium chloride and preparation, the apex of the heart was inserted in 10% buffered formalin (pH = 7.2) and then fixed in paraffin. Then, 5 μm slices were stained with

Table 1. Primer sequences

Gene	Forward	Reverse
B2m	CGTGATCTTTCTGGTGCTTGTC	GGAAGTTGGGCTTCCCATTCT
Bax	GAGGATGATTGCTGATGTGGATA	CAGTTGAAGTTGCCGTCTG
BCL2	GGAGCGTCAACAGGGAGATG	ACAGCCAGGAGAAATCAAACAGA

hematoxylin and eosin (H&E) and presented to an expert pathologist who was blinded to the study in order to assess histology and neutrophil infiltration as well as edema. A Ci-E camera (Nikon, Tokyo, Japan) with × 100 microscopic magnifications was utilized to analyze the sections at different views (23).

Statistical analysis

The obtained data were presented as means ± standard deviations. GraphPad Prism 8.1 software was used for data analysis. One-way analysis of variance (ANOVA) and Tukey's post hoc test were applied to analyze the data. Values of $P < 0.05$ were considered statistically significant.

Results

Limonene reduced creatine kinase-MB, creatine phosphokinase, and lactate dehydrogenase levels

The measured CK-MB level was increased after ischemia induction [$P < 0.001$; ISO-I (3.73 ± 0.560) versus the healthy group (0.888 ± 0.317)], indicating muscular damage (Table 2). Based on the results in Table 2, the LIM10 (3.94 ± 0.527) did not significantly decrease the CK-MB compared to the ST counterpart ($P = 0.92$). LIM20 (2.84 ± 0.323) and LIM40 (2.09 ± 0.417) markedly attenuated the CK-MB level ($P = 0.01$ and $P < 0.001$, respectively, versus the ST counterpart). Another indicator of cardiomyocyte destruction, CPK, noticeably increased after the induction of ischemia by ISO [$P < 0.001$; ISO-I (901 ± 72.2) versus the healthy group (397 ± 33.5)]. However, the results (Table 2) revealed that LIM10 (687 ± 95.3), LIM20 (596 ± 58.3), and LIM40 (491 ± 40.6) significantly decreased the CPK, alleviating tissue destruction caused by ischemia ($P < 0.001$ for all doses versus the ST counterpart). The measured LDH level was increased after ischemia induction [$P < 0.001$; ISO-I (1992 ± 176) versus the healthy group (821 ± 94.1)]. Likewise, LIM 10 (1545 ± 288), LIM 20 (1272 ± 281), and LIM 40 (1009 ± 139) reduced LDH due to ischemic-related damage ($P = 0.008$, $P < 0.001$, and $P < 0.001$, respectively, versus the ST group).

Limonene increased total antioxidant capability in the serum and heart samples

Cardiac and serum-related TAC were estimated using the FRAP assay. Ischemia in the ISO-I group (324 ± 45.0) diminished TAC in the heart tissue ($P < 0.001$) compared to the healthy group (588 ± 64.5). Based on the data in Table 3, similar findings were obtained for TAC of the serum in the healthy group versus the ISO-I group (388 ± 45.9 versus 202 ± 23.0, respectively, $P < 0.001$). LIM 10 (251 ± 25.5), LIM 20 (268 ± 25.4), and LIM 40 (293 ± 11.4) achieved an increase in serum TAC ($P = 0.04$, $P = 0.003$, and $P < 0.001$, respectively, versus the ISO + ST group). Furthermore, LIM at doses of 10 (541 ± 59.5), 20 (553 ± 43.3), and 40 mg/kg (505 ± 40.7) could significantly increase TAC in the heart tissue compared to the ISO + ST group ($P < 0.001$ for all doses).

Limonene decreased the malondialdehyde level in the serum and heart samples

The results demonstrated a significant rise in MDA levels through the ischemia in the serum samples [$P < 0.001$; ISO-I (65.6 ± 11.0) versus the CT group (28.3 ± 4.75), Table 4]. The data in Table 4 confirm that LIM 10 (42.5 ± 6.60), LIM 20 (38.5 ± 6.44), and LIM 40 (34.0 ± 5.33) reduced the serum MDA ($P < 0.001$ for all doses versus the ISO + ST group). Based on the results, a significant increase was found in the MDA levels through the ischemia in the

Table 2. Effects of LIM on the serum levels of CK-MB, CPK, and LDH among the experimental groups

Group	CK-MB (Unit/Liter)	*P* value	CPK (Unit/Liter)	*P* value	LDH (Unit/Liter)	*P* value
CT + ST	0.888 ± 0.317		397 ± 33.5		821 ± 94.1	
ISO + ST	3.73 ± 0.560 ***	< 0.001	901 ± 72.2***	< 0.001	1992 ± 176***	< 0.001
ISO + LIM 10	3.94 ± 0.527	0.92	687 ± 95.3###	< 0.001	1545 ± 288##	0.008
ISO + LIM 20	2.84 ± 0.323#	0.01	596 ± 58.3###	< 0.001	1272 ± 281#####	< 0.001
ISO + LIM 40	2.09 ± 0.417###	< 0.001	491 ± 40.6###	< 0.001	1009 ± 139###	< 0.001

Note. Values are presented as means ± standard deviations. The statistical analysis involved using a one-way ANOVA, followed by Tukey's post-test. Significance levels were denoted as ***$P < 0.001$ in comparison to the saline-treated control group and #$P = 0.01$, ##$P = 0.008$, and ###$P < 0.001$ compared to the saline-received ISO group. LIM: Limonene; CT: Control; ISO: Isoproterenol; ANOVA: Analysis of variance; CPK: Creatine phosphokinase; LDH: Lactate dehydrogenase; CK-MB: Creatine kinase-MB; ST: Saline treatment.

Table 3. Effects of LIM on TAC in the heart and serum samples among the experimental groups

Group	TAC Heart (mmol/mg Protein)	*P* value	TAC Serum (mmol/mg Protein)	*P* value
CT + ST	588 ± 64.5		388 ± 45.9	
ISO + ST	324 ± 45.0***	< 0.001	202 ± 23.0***	< 0.001
ISO + LIM 10	541 ± 59.5###	< 0.001	251 ± 25.5#	0.04
ISO + LIM 20	553 ± 43.3###	< 0.001	268 ± 25.4##	0.003
ISO + LIM 40	505 ± 40.7###	< 0.001	293 ± 11.4###	< 0.001

Note. Values are presented as means ± standard deviations. The statistical analysis involved using a one-way ANOVA, followed by Tukey's post-test. Significance levels were denoted as ***$P < 0.001$ in comparison to the saline-treated control group and #$P = 0.04$, ##$P = 0.003$, and ###$P < 0.001$ in comparison to the saline-received isoproterenol group. LIM: Limonene; CT: Control; ISO: Isoproterenol; TAC: Antioxidant capability; ANOVA: Analysis of variance; ST: Saline treatment.

heart samples [$P<0.001$; the ISO-I group (104 ± 8.70) versus the CT group (65.3 ± 7.46)]. Similar findings to the serum were observed for the heart's MDA levels, so that LIM 10 (62.5 ± 7.42), LIM 20 (65.5 ± 6.67), and LIM 40 (56.4 ± 11.5) significantly reduced the cardiac level of MDA ($P<0.001$ for all doses versus the ISO + ST group).

Limonene enhanced anti-apoptotic function by upregulating BCL2 and downregulating BAX

Based on the results (Table 5), ISO-I rats indicated an increased expression of the *BAX* gene (2.75 ± 0.124) versus the CT + ST group (1.08 ± 0.0938) ($P<0.001$) and decreased expression of the *BCL2* gene (0.880 ± 0.118) in comparison to the ST control group (1.74 ± 0.178) ($P<0.001$). LIM at doses of 20 (1.40 ± 0.152, $P<0.001$) and 40 mg/kg (1.65 ± 0.0598, $P<0.001$) notably enhanced the anti-apoptotic gene in the heart by overexpressing the *BCL2* gene compared with the ISO + ST group. Simultaneously, LIM at a dose of 10 mg/kg (0.863 ± 0.379) did not change *BCL2* gene expression compared to the ISO + ST group ($P=0.99$). Following LIM administration, pro-apoptotic protein BAX was downregulated compared to its expression in the ST counterpart [$P=0.003$, $P<0.001$, and $P<0.001$ for LIM 10 (2.29 ± 0.267), LIM 20 (1.93 ± 0.288), and LIM 40 mg/kg (1.38 ± 0.114), respectively].

Limonene reconstructed ischemic-associated histopathological changes

According to figures presenting microscopic features of sections stained with H&E, the ISO-I group showed increased inflammatory cell infiltration, intracellular space, total collagen content, and fibrosis. LIM therapy could reverse these values to a level comparable to the control group (Figure 1).

Discussion

Monoterpenes are among the most esteemed plant-derived compounds, with a great tendency for researchers to figure out their anti-ischemic, antioxidant, and anti-inflammatory features more exclusively in the recent decade. The current study assessed the effect of LIM administered at defined doses of 10 mg/kg, 20 mg/kg, and 40 mg/kg in rats experiencing a myocardial infarction-like condition using ISO. Based on the results, LIM enhanced the TAC (an anti-oxidative index) and anti-apoptotic function, while it reduced MDA following an ischemic crisis. LIM improved the structural degradation of cardiomyocytes due to ischemia, which was established by a reduction in cardiomyocyte damage indices, including CK-MB, CPK, and LDH. Compounds released by herbs such as LIM are well-liked to be administered due to their anti-ischemic advantages before the occurrence of myocardial injury or clot-related stenosis (14). In the same way, our study focused on LIM as a cardioprotective potential in ISO-I rats mimicking an acute heart crisis. It was found that the administration of LIM lowers muscle injury biomarkers and reverses the deleterious histopathological changes in the heart. It is assumed that a local inflammation would occur due to an insufficiency to preserve blood flow in the coronary arteries and myocardium. This inflammation mediates the apoptosis and necrosis in cardiomyocytes during a myocardial infarction-like disaster (16,20).

ISO is a pure, nonselective beta agonist medication with superior inotropic and chronotropic properties on the cardiac muscle. As previously described, it induces a perfusion supply/cardiac imbalance at the specific dosage, causing tissue ischemia. Fundamentally, ischemia considerably produces ROS and inflammatory mediators by constituting oxidative stress. Indeed, oxidative stress is

Table 4. Effects of LIM on MDA in the serum and heart samples among the experimental groups

Group	MDA heart (nmol/mg protein)	*P* value	MDA serum (nmol/mg protein)	*P* value
CT + ST	65.3 ± 7.46		28.3 ± 4.75	
ISO + ST	104 ± 8.70***	< 0.001	65.6 ± 11.0***	< 0.001
ISO + LIM 10	62.5 ± 7.42$^{\#\#\#}$	< 0.001	42.5 ± 6.60$^{\#\#\#}$	< 0.001
ISO + LIM 20	65.5 ± 6.67$^{\#\#\#}$	< 0.001	38.5 ± 6.44$^{\#\#\#}$	< 0.001
ISO + LIM 40	56.4 ± 11.5$^{\#\#\#}$	< 0.001	34.0 ± 5.33$^{\#\#\#}$	< 0.001

Note. Values are provided as means ± standard deviation. Statistical analysis involved using a one-way ANOVA, followed by Tukey's post-test. Significance levels were represented as $^{***}P<0.001$ in comparison to the saline-treated control group and $^{\#\#\#}P<0.001$ compared to the saline-received isoproterenol group. ANOVA: Analysis of variance; LIM: Limonene; CT: Control; ISO: Isoproterenol; ST: Saline treatment; MDA: Malondialdehyde.

Table 5. Effects of LIM on *BCL2* and *BAX* gene expressions in the heart samples among the experimental groups

Group	BCL2 relative gene expression	*P* value	BAX relative gene expression	*P* value
CT + ST	1.74 ± 0.178		1.08 ± 0.0938	
ISO + ST	0.880 ± 0.118***	< 0.001	2.75 ± 0.124***	< 0.001
ISO + LIM 10	0.863 ± 0.379	= 0.99	2.29 ± 0.267$^{\#\#}$	= 0.003
ISO + LIM 20	1.40 ± 0.152$^{\#\#\#}$	< 0.001	1.93 ± 0.288$^{\#\#\#}$	< 0.001
ISO + LIM 40	1.65 ± 0.0598$^{\#\#\#}$	< 0.001	1.38 ± 0.114$^{\#\#\#}$	< 0.001

Note. Significance levels were denoted as $^{***}P<0.001$ in comparison to the saline-treated control group and $^{\#\#}P$: 0.003 and $^{\#\#\#}P<0.001$ in comparison to the saline-received isoproterenol group. LIM: Limonene, CT: Control, ISO: Isoproterenol; ST: Saline treatment; ANOVA: Analysis of variance. Values are presented as means ± standard deviations. Statistical analysis involved using a one-way ANOVA, followed by Tukey's post-test.

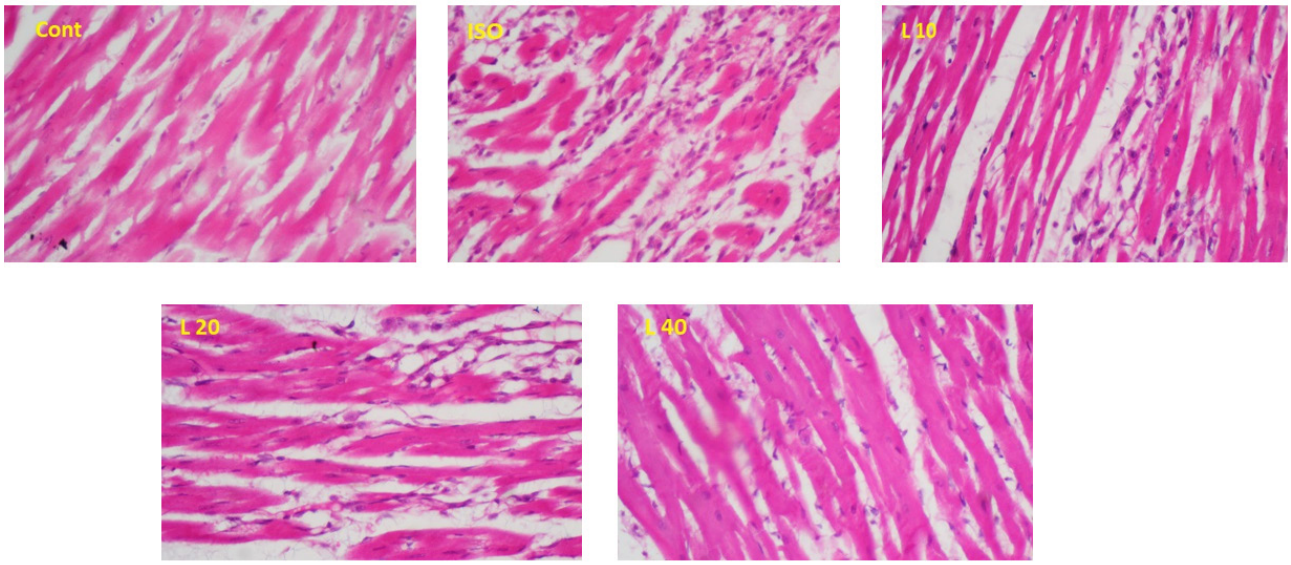

Figure 1. LIM alleviated histological myocardial changes at its administered doses in ISO-I/myocardial damage in rats. The representative histoarchitecture of the tissue (× 100) stained with H&E shows normal histology (Cont). ISO-I generated a considerable inflammatory cell infiltration with deeply stained pyknotic nuclei and increased interstitial spaces and myofiber malalignment (ISO). Treatment with L10 improved the myofiber disarrangement, while the infiltrated cells with extremely stained nuclei were still observable (L 10). A dose-dependent increase of LIM could enhance myofiber arrangement and muscle structure, attenuate inflammatory cell infiltration, and restore interstitial space (L 20 and L40). L: Limonene; Cont: control normal.

the "master key" to forming a vicious cycle, consequently generating inflammation-related injury (24,25). LIM boosts antioxidant function by different mechanisms. In particular, it has been reported that LIM restores antioxidant-related components, including superoxide dismutase and glutathione peroxidase, through oxidized Ca^{2+}calmodulin-dependent kinase II inhibition (14,20). From a different point of view, the results of this study demonstrated the promotion of antioxidant activity by measuring the reducing power of ferric ions. TAC was increased in animals that received LIM. Apparently, LIM encourages the antioxidant content in a dose-dependent manner. Worthwhile advantages in promoting antioxidant potential were observed when LIM was administered at 10 mg/kg, 20 mg/kg, and 40 mg/kg. LIM at a lesser concentration of 10 µmol, as presented in the study conducted by Durço et al, decreased the dihydroethidium fluorescence representative, total sulfhydryl, and carbonyl content; however, the FRAP assay did not indicate significant improvements at this dose. LIM at this concentration could also suppress lipid peroxidation by diminishing MDA (20), which conforms to our findings for doses of 10 mg/kg, 20 mg/kg, and 40 mg/kg of LIM at the cardiac level of MDA. On the other hand, serum MDA was simultaneously assessed in this study, revealing a decrease after LIM treatment.

MDA arises when polyunsaturated fatty acid chains break down in the cell membrane. It has recently emerged as a diagnostic biomarker in ischemia, particularly for the evaluation of IHD, heart failure, and post-stroke complications (26-28). In line with our findings, Hung et al appointed MDA-oxidized low-density lipoprotein, an oxidative product resulting from assembled MDA onto an apolipoprotein component, to a valuable biomarker that indicated a positive arterial stiffness possibility and a cardio-ankle vascular index predictor in coronary artery bypass graft patients (29). Based on the obtained results in the current study, it is reasonable to emphasize that LIM can improve atherosclerosis in peripheral circulation throughout the body by reducing the adverse effects of lipid peroxidation.

Fundamentally, a disturbed blood supply/cell demand balance is almost always the foremost reason for tissue ischemia, causing the release of injured muscle-related biomarkers, including CK-MB, CPK, and LDH. Their release into the bloodstream is facilitated by the lipid peroxidation of injured cardiomyocyte membrane components caused by ischemia and oxidative stress (30,31). Among these markers of muscle degradation, increased serum levels of CK-MB and troponin are the most widely accepted and trustworthy indicators of cardiac injury (32). In practice, the levels of these biomarkers are assigned as heart crisis condition alarms seeking an urgent or emergent medical intervention. LIM (at 200 mg/kg concentration) has been observed to decrease CPK, CK-MB, and troponin-T levels by preventing the deterioration of the cardiomyocyte cell membrane caused by the cardiotoxic substance of carbon tetrachloride in Wistar rats (16). Based on the result of this study, LIM at doses of 20 mg/kg and 40 mg/kg could lessen cardiac injury induced by ISO, cell damage, and histopathological changes related to ischemia. In addition to CK-MB and CPK, LDH is announced as a diagnostic biomarker linked to the destruction of various cells, including red blood cells, hepatocytes, and cardiomyocytes (30). LIM decreased serum LDH levels when administered at 20 mg/kg and 40 mg/kg doses.

Biochemical changes and histopathological features co-

occur over the course of ischemia. ISO represented the signs of tissue breakup as increased intracellular space, inflammatory cell infiltration, malalignment of myofibrils, and vacuolization changes (14). LIM might prevent myocardial injury by minimizing discontinuity, reducing cellular infiltration, and preserving the arrangement of myofibrils in dose-dependent relationships.

As mentioned earlier, pathologic cellular death and cardiomyocyte apoptosis, alongside oxidative stress, are two leading actors forming histopathologic and laboratory features of ischemic damage (33). Concerning apoptosis, it was found that LIM effectively fostered the anti-apoptotic function by increasing the *BCL2* gene expression. Similar to our findings, Younis et al suggested that pretreatment with LIM (50 mg/kg) could amplify the relative expression of anti-apoptotic protein BCL2 and attenuate BAX2 expression (34).

Based on the results obtained from the current animal study, it is suggested that more detailed pharmacodynamics and pharmacokinetic studies focus on the possible cardioprotective effects of LIM in rats to find exact underlying mechanisms. If promising results are obtained, clinical studies should be conducted to investigate the impact of LIM in humans.

This study had some limitations. Apart from assessing the antioxidant function, other variables of ROS-activated cell death should also receive attention, and a more specific biomarker such as troponin should be measured following LIM administration in ISO-I rats. We approvingly recommend further evaluations to explore how LIM boosts its anti-apoptotic function.

Conclusion

Overall, it was found that LIM, a beneficial monoterpene, could, at least, partially ameliorate ischemic-related cardiac injury induced by ISO through its antioxidant and anti-apoptotic potentials.

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Competing Interests

The authors declare that there is no conflict of interests.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was approved by the Ethics Committee on Animal Investigation at Shahrekord University of Medical Sciences (with the ethical code: IR.SKUMS.REC.1398.160). Additionally, housing conditions and experiments all complied with the rules issued by the ARRIVE guidelines and Guide for the Care and Use of Laboratory Animals (9th edition, National Academies Press). Full efforts were made to reduce discomfort in animals.

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