Original Article

Asian Pacific Journal of Tropical Biomedicine

apjtb.org

doi: 10.4103/2221-1691.374231

Impact Factor® 1.51

Cardioprotective effects of *Pinus eldarica* bark extract on adrenaline-induced myocardial infarction in rats

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ABSTRACT

Objective: To investigate the effect of *Pinus eldarica* bark extract on adrenaline-induced myocardial infarction.

Methods: Hydroalcoholic extract was prepared using maceration method and its total phenolic content was determined using the Folin-ciocalteu method. Pretreatment was done by oral administration of 100, 200, and 400 mg/kg *Pinus eldarica* bark extract for 16 days in male Wistar rats. Injection of adrenaline (2 mg/kg, *s.c.*) was performed on the 15th and 16th days for induction of myocardial infarction. Lead Ⅱ EEG was recorded. Serum cardiac marker enzymes and antioxidative parameters were evaluated and a histopathological examination of heart tissues was performed.

Results: Pretreatment with *Pinus eldarica* bark extract especially at its high doses significantly lowered the ST-segment elevation, improved heart rate, and decreased RR interval in ECG pattern of rats with adrenaline-induced myocardial infarction. It declined serum markers of heart damage including aspartate aminotransferase, lactate dehydrogenase, and creatine phosphokinase-MB, and also decreased lipid peroxidation marker, and heart weight while raising total antioxidant capacity and considerably improved histopathological alterations of the heart induced by adrenaline.

Conclusions: *Pinus eldarica* bark extract shows beneficial cardioprotective and antioxidant effects against adrenaline-induced myocardial infarction. It can be further explored as a potential treatment for myocardial infarction.

KEYWORDS: Adrenaline; Antioxidant; Lipid peroxidation; Myocardial infarction; *Pinus eldarica*

1. Introduction

Cardiovascular diseases (CVD) are the most important reason for death in the world. According to global statistics, around 17.9 million people die worldwide every year due to cardiovascular problems, which includes 32% of all deaths. Heart attack and stroke are responsible for 85% of this mortality[1]. Heart attack or myocardial infarction (MI) is a critical medical emergency that may lead to heart failure or death if not quickly and properly managed. Current treatment approaches such as drugs and surgery are not able to completely restore the structure and function of the ischemic heart. Cardioprotective interventions that prevent heart attack or at

Significance

Pines belonging to the *Pinus* genus have shown beneficial cardiovascular effects. This study demonstrates the cardioprotective effects of *Pinus eldarica* bark extract against adrenaline-induced myocardial infarction through reducing the enzyme markers of heart damage and lipid peroxidation and improving electrocardiogram, heart histopathology and antioxidant defense.

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How to cite this article: Safaeian L, Haghighatian Z, Zolfaghari B, Amindeldar M. Cardioprotective effects of *Pinus eldarica* bark extract on adrenaline-induced myocardial infarction in rats. Asian Pac J Trop Biomed 2023; 13(4): 148-155.

Article history: Received 14 March 2023; Revision 31 March 2023; Accepted 3 April 2023; Available online 27 April 2023

least reduce myocardial tissue damage and improve heart adaptation in post-MI conditions are of great importance and concern[2,3].

Oxidative stress, mitochondrial dysfunction, inflammatory process, increased intracellular calcium, necrosis, and apoptosis have been proposed in the pathogenesis of cardiovascular damage during MI[4]. Oxidative stress through excess production of reactive oxygen species and lipid peroxidation in the cell membrane is involved in the permanent injury to the myocardial membrane and loss of cardiac function[5]. Many investigations have shown the promising role of natural polyphenolic compounds in CVD[6]. Pines belonging to the *Pinus* genus are rich in polyphenols and proanthocyanidins with beneficial cardiovascular effects[7,8]. *Pinus eldarica* (*P. eldarica*) Medw., one of the fast-growing pines from Pinaceae family is native to the Middle East and broadly grows in Iran[9]. The bark extract of *P. eldarica* has high polyphenolic content with antioxidant and cytoprotective effects against oxidative damage in human endothelial cells, and capability to improve hyperlipidemia, atherosclerosis, diabetes, and inflammatory conditions[10–13]. The current study aimed to examine the possible helpful effects of the bark extract of *P. eldarica* on adrenaline-induced MI in rats.

2. Materials and methods

2.1. Plant material and preparation of extract

The barks of *P. eldarica* were collected from pine stands in November 2021 at Isfahan city (Isfahan Province) in the center of Iran. The pine sample was authenticated with a voucher specimen (No. 3318) stored in the Herbarium at the Department of Pharmacognosy.

The hydroalcoholic extract was prepared by maceration technique. Briefly, the dried pine materials were ground and macerated with ethanol (70%) at room temperature for 72 h three times. After filtration, the resultant extract was concentrated by removing the solvent using a vacuum rotary evaporator under low pressure. The extract powder was acquired through freeze-drying and reserved at –20 ℃. Different doses of hydroalcoholic extract of pine bark were dissolved in normal saline and administered orally using an intragastric tube.

2.2. Determination of total phenolic content

In our previous phytochemical analysis through high pressure liquid chromatography, polyphenolic compounds including catechin (3.41%), ferulic acid (2.27%), taxifolin (1.95%) and caffeic acid (1.62%) were found in the bark extract of *P. eldarica*[9]. In this study, Folin-Ciocalteu assay was done for determining the amount of total phenolic compounds and standardization of the hydroalcoholic extract of *P. eldarica* bark based on the content of phenolics. A mixture of phosphomolybdate and phosphotungstate solution was used as the Folin-Ciocalteu reagent in this colorimetric method. Briefly, the pine or standard samples were mixed with sodium carbonate (20%) and then incubated with diluted Folin-Ciocalteu reagent for 120 min. The UV absorbance was detected using a spectrophotometer at 765 nm. The total phenolic content of samples was assessed using a standard curve depicted by different concentrations of gallic acid and specified in terms of mg of gallic acid equivalent (GAE)/g of the dried pine bark extract[14].

2.3. Animals and experimental design

Male adult Wistar rats, weighing (250 ± 20) g, were acquired from the animal house of the School of Pharmacy and Pharmaceutical Sciences. The rats were maintained under room temperature of 20-25 ℃ and a 12 h light/12 h dark cycle with free access to water and standard food. Animals were adapted under experimental environment for 1 week before the beginning of the experiment. The experimental practice was done in accordance with the international guidelines for laboratory animal use and care.

The adrenaline-induced MI model was developed by administration of adrenaline (Iran Hormone Pharmaceutical Co., Tehran, Iran) 2 mg/kg subcutaneously (*s.c.*) for 2 consecutive days (24 h apart)[15]. Rats were randomly divided into 6 groups with 6 rats in each group as follows: The first group as the normal control received oral administration of the vehicle (normal saline) for 16 d and *s.c.* injection of normal saline on days 15 and 16. The second group as the extract control received only *P. eldarica* bark extract orally at the dose of 400 mg/kg for 16 d. In the third group as the MI control group, adrenaline (2 mg/kg/day, *s.c.*) was administered on days 15 and 16. In groups 4-6 as the test groups, rats were treated with 100, 200, or 400 mg/kg of *P. eldarica* bark extract orally for 16 d and received *s.c.* injection of adrenaline on days 15 and 16. The doses of *P. eldarica* bark extract were selected based on the previous studies[11,13].

Animals were weighed at the start of the experiment and then every other day. After 24 h of adrenaline administration, rats were anesthetized and lead Ⅱ electrocardiograph (ECG) was recorded using computerized data acquisition eWave system and analyzed with eProbe software (Science Beam; Parto Danesh Co., Iran). Heart rate (HR) and RR interval were recorded and alteration of ST-segment was evaluated. Then, blood samples were taken by retro-orbital technique from the anesthetized rats and serum was separated for evaluation of biochemical parameters of heart damage and oxidative stress. After scarifice under CO_2 exposure, hearts were removed, weighed, and then fixed in 10% formalin solution and used for histopathological examination after additional processing.

2.4. Biochemical assay

Serum levels of creatine phosphokinase-MB (CK-MB), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) were estimated spectrophotometrically at 340 nm using the commercial biochemical kits (Pars Azmoon Co., Iran)[16].

2.5. Lipid peroxidation assay

The level of serum malondialdehyde (MDA) was examined carefully for assessment of lipid peroxidation *via* spectrophotometrically thiobarbituric acid reactive substances test using a standard kit (Hakiman Shargh Research Co., Isfahan, Iran). The absorbance of samples was measured at 532 nm and the content of lipid peroxides was expressed as MDA equivalents in μM[17].

2.6. Total antioxidant capacity assay

The ferric reducing antioxidant power (FRAP) test was used for evaluation of the antioxidant power in serum samples using a standard kit (Hakiman Shargh Research Co., Isfahan, Iran). This spectrophotometrical assay estimates the reduction of ferric-tripyridyl triazine complex to ferrous form at 570 nm. The FRAP levels were expressed as mM of ferrous sulphate equivalents using a standard curve of ferrous sulphate[18].

2.7. Histopathological examination

The heart samples were processed and embedded in paraffin blocks. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and inspected under a light microscope for histopathological alterations. Finally, tissue images of heart sections were obtained using a digital camera.

2.8. Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) and subjected to one-way analysis of variance (ANOVA) and then Tukey *post-hoc* test *via* the Statistical Package for Social Sciences (SPSS software version 25.0) for statistical evaluation. The significant difference was set at P value<0.05.

2.9. Ethical statement

The research procedures were approved by the Institutional Research Ethics Committee of Isfahan University of Medical Sciences with ethics approval ID: IR.MUI.RESEARCH.REC.1400.353.

3. Results

3.1. Total phenolic content

Total phenolic content of *P. eldarica* bark extract was determined as (560.65 ± 44.00) mg GAE/g of the dried plant extract.

3.2. Effect of P. eldarica bark extract on ECG parameters

As shown in Figure 1, administration of adrenaline (2 mg/kg) for 2 successive days caused obvious changes in ECG pattern such as elevation of ST segment. Moreover, an increase in RR interval by 36.42% and a decrease in HR by 26.82% were observed in rats receiving adrenaline compared with the control group (P<0.001) (Table 1).

Pretreatment with *P. eldarica* bark extract at the doses of 200 and 400 mg/kg significantly lowered the ST-segment elevation and improved the HR (P<0.05 and P<0.001 at 200 and 400 mg/kg, respectively) in rats with adrenaline-induced MI. *P. eldarica* extract also notably decreased RR interval at a dose of 400 mg/kg (P<0.01) (Figure 1 and Table 1).

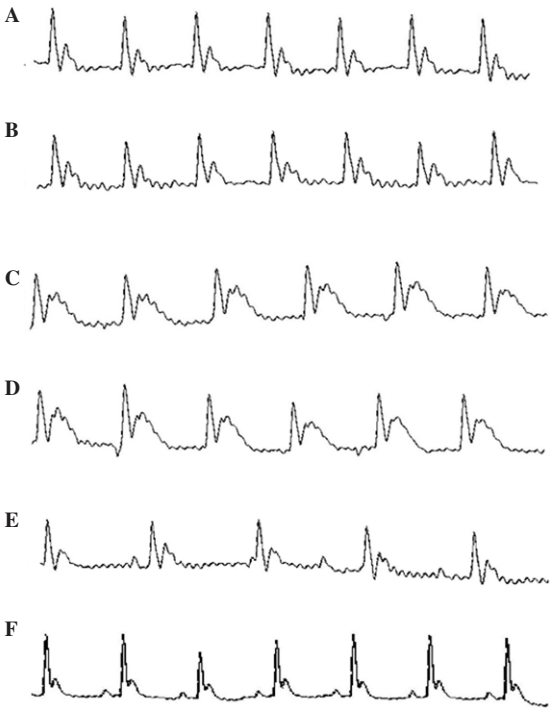

Figure 1. Representative electrocardiographs traces of lead Ⅱ for normal rats (A); rats treated with *Pinus eldarica* (*P. eldarica*) bark extract (B); rats with adrenaline-induced myocardial infarction (C); rats with adrenaline-induced myocardial infarction treated with *P. eldarica* bark extract at doses of 100 (D), 200 (E), and 400 mg/kg (F).

Table 1. Effect of hydroalcoholic extract of *P. eldarica* bark on electrocardiogram parameters in rats with adrenaline-induced myocardial infarction (MI).

Groups	Heart rate (BPM)	RR-interval (ms)
Normal control	358.3 ± 7.5	167.2 ± 8.1
P. eldarica control (400 mg/kg)	340.2 ± 6.8	176.3 ± 9.2
MI control	262.2 ± 8.2$^{###}$	228.1 ± 9.9$^{###}$
MI + *P. eldarica* (100 mg/kg)	274.1 ± 9.1	218.8 ± 7.3
MI + *P. eldarica* (200 mg/kg)	278.9 ± 3.5*	215.4 ± 7.1
MI + *P. eldarica* (400 mg/kg)	320.4 ± 8.5***	187.2 ± 8.3**

Values are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA, followed by Tukey *post hoc* analysis. $^{###}P$<0.001 versus the normal control; $^{*}P$<0.05, $^{**}P$<0.01, and $^{***}P$<0.001 versus the MI control. BPM: beat per minute; ms: millisecond.

3.3. Effect of P. eldarica bark extract on biochemical parameters

Figure 2 indicates the effect of hydroalcoholic extract of *P. eldarica* bark on biochemical parameters in adrenaline-induced MI. Following adrenaline injection, the serum level of CK-MB activity was increased to (319.20 ± 38.00) IU/L compared to the normal control group (156.00 ± 0.27) IU/L (P<0.05). Pretreatment with different doses of *P. eldarica* extract declined the activity of this enzyme, especially at a dose of 400 mg/kg of extract (147.20±24.00) IU/L when compared to the MI control group (P<0.05). A significant elevation in the activity of LDH was observed after administration of adrenaline (P<0.001), which was reduced by treatment with *P. eldarica* bark extract. Adrenaline also caused a significant rise in the serum activity of AST (P<0.001). *P. eldarica* extract (200 and 400 mg/kg) significantly reduced AST level as compared to MI control rats (P<0.001). Treatment with 400 mg/kg of *P. eldarica* extract alone in normal rats did not change the levels of biochemical parameters.

3.4. Effect of P. eldarica bark extract on lipid peroxidation and total antioxidant capacity

As shown in Figure 3, adrenaline resulted in a high increase in the content of MDA when compared to the normal control group (P<0.001). Treatment with *P. eldarica* bark extract at the doses of 100, 200, and 400 mg/kg markedly attenuated the MDA level (P<0.05, P<0.01, and P<0.001, respectively).

The FRAP value was notably reduced in serum of adrenaline-treated rats (P<0.05). *P. eldarica* bark extract significantly raised the FRAP value at a dose of 400 mg/kg compared to the MI control rats (P<0.05) (Figure 3).

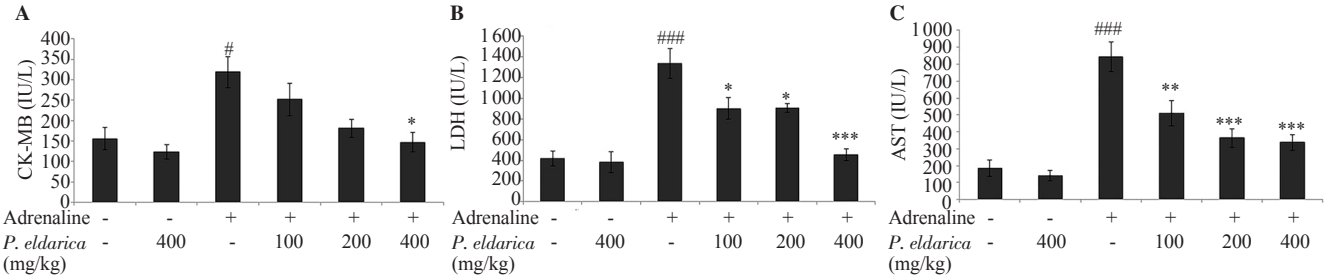

Figure 2. Effect of *P. eldarica* bark extract (100-400 mg/kg) on serum CK-MB (A), LDH (B), and AST (C) in rats with adrenaline-induced MI. Values are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA, followed by Tukey *post hoc* analysis. $^{#}P$<0.05 and $^{###}P$<0.001 versus the normal control; $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 versus the MI control. CK-MB: creatine phosphokinase-MB, LDH: lactate dehydrogenase, AST: aspartate aminotransferase.

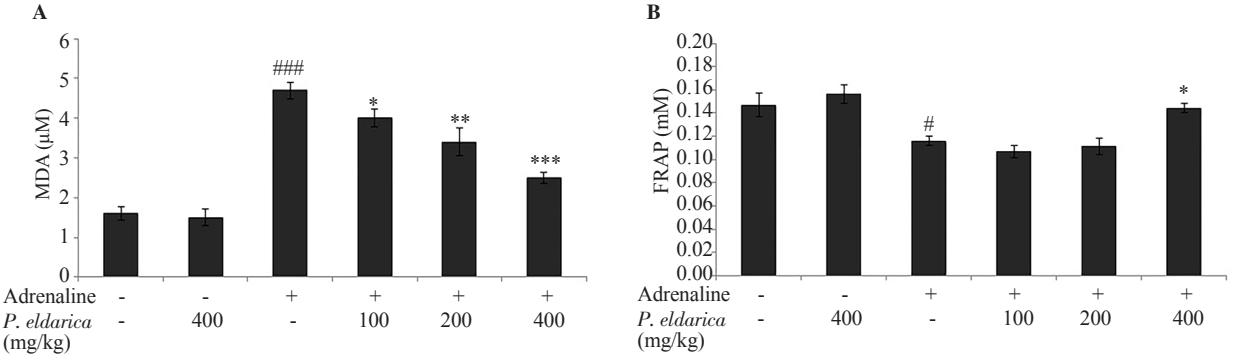

Figure 3. Effect of *P. eldarica* bark extract (100-400 mg/kg) on serum MDA level (A) and FRAP value (B) in rats with adrenaline-induced MI. $^{#}P$<0.05 and $^{###}P$<0.001 versus the normal control; $^{*}P$<0.05, $^{**}P$<0.01 and $^{***}P$<0.001 versus the MI control. MDA: malondialdehyde, FRAP: ferric reducing antioxidant power.

Table 2. Effect of hydroalcoholic extract of *P. eldarica* bark on body weight and relative heart weight of rats with adrenaline-induced MI (%).

Groups	Body weight changes	Relative heart weight
Normal control	9.61 ± 1.50	0.447 ± 0.010
P. eldarica control (400 mg/kg)	7.59 ± 1.10	0.425 ± 0.012
MI control	8.18 ± 0.60	0.553 ± 0.013$^{###}$
MI + *P. eldarica* (100 mg/kg)	8.29 ± 0.80	0.548 ± 0.014
MI + *P. eldarica* (200 mg/kg)	6.63 ± 0.50	0.431 ± 0.013***
MI + *P. eldarica* (400 mg/kg)	7.92 ± 0.70	0.420 ± 0.008***

Values are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA, followed by Tukey *post hoc* analysis. $^{###}P$<0.001 versus the normal control; $^{***}P$<0.001 versus the MI control.

3.5. Effect of P. eldarica bark extract on body and heart weight changes

As seen in Table 2, the weight of all rats was increased during the test period and no significant difference in the body weight changes was observed between different groups. The adrenaline-induced MI resulted in a significant rise in heart weight (P<0.001). However, *P. eldarica* bark extract markedly lowered heart weight at doses of 200 and 400 mg/kg (P<0.001).

3.6. Effect of P. eldarica bark extract on heart histopathology

In morphological examination of the heart section, a normal structure of cardiomyocytes without any inflammation was observed in the heart of control rats (Figure 4A) and rats treated only with *P. eldarica* bark extract (400 mg/kg) (Figure 4B). Conversely, clear histological changes including infiltration of inflammatory cells, degeneration of myocytes (rupture and vacuolization), as well as areas of hemorrhage and congestion of blood vessels were demonstrated after injection of 2 mg/kg adrenaline for 2 successive days in MI control rats (Figure 4C). Administration of *P. eldarica* bark extract at the dose of 100 mg/kg had no significant effect (Figure 4D), however, 200 mg/kg (Figure 4E) and 400 mg/kg (Figure 4F) improved histopathological alterations of the heart induced by adrenaline.

4. Discussion

The current study examined the effects of *P. eldarica* bark extract pretreatment on parameters of cardiac damage in a MI rat model. The adrenaline-induced MI was confirmed by irregularities in ECG pattern including elevated ST segment, prolonged RR interval and

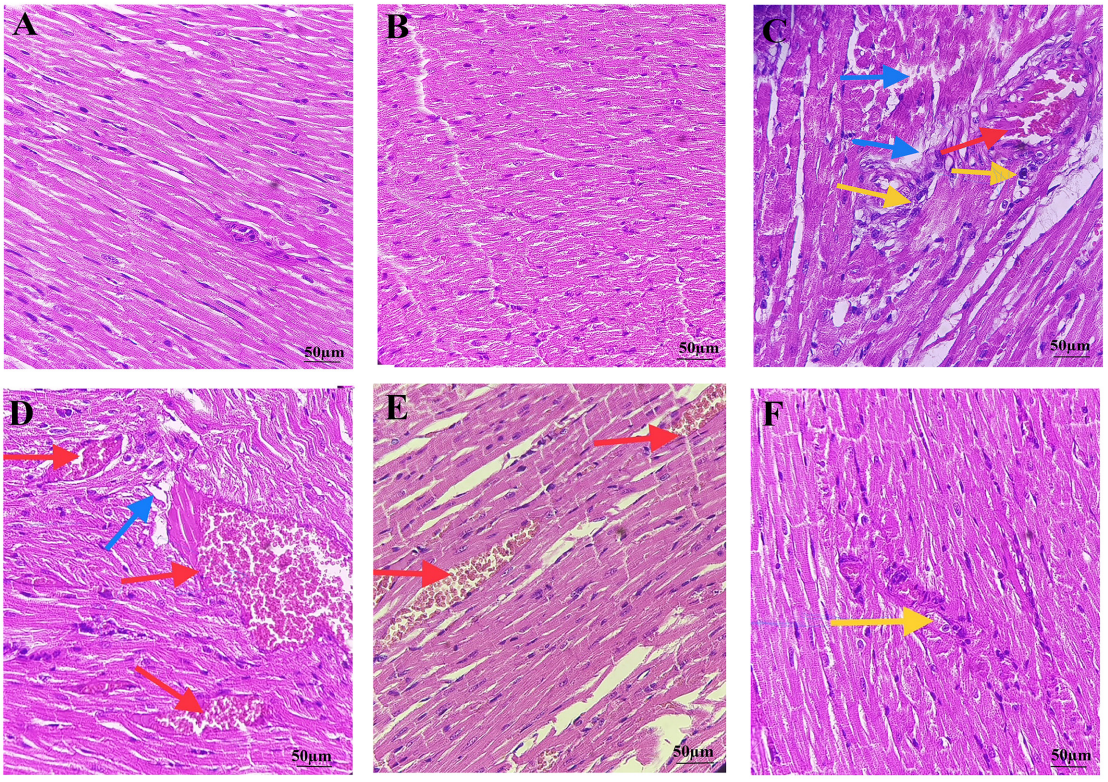

Figure 4. Representative H & E sections of heart tissue for normal control (A); *P. eldarica* bark extract alone treated group (B); adrenaline-induced MI (C); *P. eldarica*-treated MI rats at 100 (D), 200 (E) and 400 mg/kg (F); ×400 magnification. Arrows indicate hemorrhage and congestion (red), inflammation (yellow) and degeneration of myocytes (blue).

declined HR, raised serum levels of CK-MB, LDH, and AST, cardiac weight gaining, histopathological alterations, and oxidative damage. MI induced by catecholamines including adrenaline and isoproterenol in mice and rats is used as a standard model to evaluate the potential of compounds with possible cardioprotective activities[17,19]. ECG abnormalities especially ST-segment elevation occur due to the difference between the ischemic and non-ischemic area indicating cell membrane dysfunction such as ischemia, cardiac necrosis, and myocardial damage[5,20]. Adrenaline, as a beta-adrenergic receptor agonist, causes myocardial hyperactivity and results in coronary artery spasm, and leads to heart ischemia by affecting alpha-adrenergic receptors. Following ischemia and infarction caused by necrosis and myocyte damage, mean arterial pressure, HR, and heart contractility are reduced[21].

Myocardial injury caused by adrenaline administration also leads to leakage of cardiac enzymes including CK-MB, LDH, and AST into the bloodstream[17]. The release of these diagnostic enzymes occurs due to the catecholamine-induced oxidative stress and overstimulation of beta-adrenergic receptors and subsequently functional and structural damages in the myocardium which lead to the disruption in the permeability and plasma membrane integrity[16].

In this study, pretreatment with hydroalcoholic extract of *P. eldarica* bark at the doses of 200 and 400 mg/kg showed protective effect against adrenaline-induced MI in rats through improving ECG pattern, reducing serum levels of CK-MB, LDH, and AST, and alleviating cardiac weight gaining and pathological changes. There was a reduction of 17.9% in RR interval and an increase of 22.2% in HR after administration of *P. eldarica* bark extract at a dose of 400 mg/kg in rats.

Beneficial cardiovascular effects have been observed in other *Pinus* species. *Pinus pinaster* bark extract (pycnogenol) regulates many risk factors of CVD such as high blood pressure, hemoglobin A1C, platelet aggregation, and hyperlipidemia[22]. Pycnogenol has also improved endothelium function in patients with coronary artery problems by reducing oxidative stress, increasing nitric oxide levels, and improving blood supply[23]. *Pinus radiata* bark extract (Enzogenol) has useful effects in improving brain, heart, and vascular function, and preventing arteriosclerosis development[24,25]. Sudjarwo and co-workers also reported cardioprotective effects of *Pinus merkusii* bark extract (100-400 mg/kg) against heart damage caused by lead acetate through modifying LDH, CK-MB, and histological alterations in rats[26]. Moreover, the anti-thrombotic activity and prevention of collagen-dependent platelet aggregation have been proved for *Pinus gerardiana* nut oil *in vitro*[27]. Regarding *P. eldarica*, antidyslipidemic activity has been reported for 200 and 400 mg/kg of bark extract through reversing hyperglycemia, hypertriglyceridemia, and hypercholesterolemia[11]. In the study of Huseini *et al*., *P. eldarica* nut extract (100 and 200 mg/kg) reduced blood cholesterol levels and improved aortic atherosclerotic involvement in hypercholesterolemic rabbits[12]. Moreover, *P. eldarica* bark extract has shown cell protective effects against oxidative damage in human endothelial cells *in vitro*[10].

Our results also showed the antioxidant activities of *P. eldarica* bark extract by improving total antioxidant capacity and declining lipid peroxidation in adrenaline-induced MI. High amounts of phenolic compounds in the bark of *P. eldarica* [(560.65 ± 44.00) mg GAE/g of extract] denote its possible beneficial effects in many disorders related to oxidative stress. Varied quantities have been reported for total phenolic content in different species of *Pinus*. In the study of Kim and his colleagues, total phenolics in the aqueous extract of *Pinus thunbergii*, *Pinus densiflora*, and *Pinus pinaster* were calculated as (192.9 ± 13.4), (524.70 ± 2.76) and (440.00 ± 2.23) mg GAE/g of extract, respectively[28]. The amounts of phenolic compounds in the plants and their antioxidant properties may be affected by the place of growth, harvesting time, techniques, and different solvents used for extraction[29]. In general, the total phenolic content in the bark of pine tree is much higher than its seeds and needle[30].

The existence of bioactive components in the bark of *P. eldarica* including polyphenolics such as taxifolin, catechin, ferulic acid, and caffeic acid, and terpenoids such as β-caryophyllene and α-pinene is accountable for the cardiovascular activities of this pine[9]. There is much evidence for antioxidant, vasorelaxant, and antihyperlipidemic effects of taxifolin[31]. Recent studies have reported the protective activities of taxifolin against cardiac injuries caused by isoproterenol or ischemia/reperfusion through diminishing inflammatory, oxidative, and apoptotic pathways and stimulating Nrf2 (nuclear factor erythroid 2-related factor 2)/HO-1 (heme oxygenase-1) signal[32,33]. Catechins also possess many beneficial impacts on cardiovascular system by counteracting hypertension, atherosclerosis, and thromboembolic events[34]. Moreover, phenolic acids have shown potent protection against various CVD risk factors[35]. In the study of Yogeeta *et al*., ferulic acid accompanied by ascorbic acid inhibited lipid peroxidation, restored antioxidant defense, and enzymatic myocardial indicator levels during MI induced by isoproterenol[36].

The major limitations of the present study included the requirement for high doses of catecholamine for induction of MI model which may be associated with other side effects and also lack of exploration of the detailed molecular mechanisms involved in the cardioprotective effect of *P. eldarica* bark extract.

In conclusion, the present findings confirmed that the hydroalcoholic extract of *P. eldarica* bark protected against adrenaline-induced MI in rats by improving ECG patterns, reducing serum levels of cardiac enzymes, attenuating pathological changes and lipid peroxidation, and improving total antioxidant capacity. Thus, *P. eldarica* bark can be explored as a potential cardioprotective treatment against MI, which needs further investigation.

Conflict of interest statement

The authors have no conflict of interest to declare.

Funding

This study was financially supported by Vice-Chancellery for Research and Technology of Isfahan University of Medical Sciences (research projects No. 3400680).

Authors' contributions

LS was responsible for the conceptualization of the study, supervision, designing the animal investigation, and editing of the manuscript; ZH contributed to the histopathological examination; BZ designed the herbal studies; MA was involved in the animal treatments, data acquisition, and preparation of the manuscript.

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