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RESEARCH ARTICLE

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Nitric oxide plays a pivotal role in cardioprotection induced by pomegranate juice against myocardial ischemia and reperfusion

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Pomegranate juice (Pg) has demonstrated cardiovascular beneficial effects. The current research intends to investigate the roles of nitric oxide (NO) and antioxidants in Pg-induced cardioprotection against ischemia and reperfusion (I/R). Isolated hearts from anesthetized rats were subjected to 30-min global ischemia followed by 120-min reperfusion. The hearts in the test groups were treated with Pg, N^G-nitro-L-arginine methyl ester (L-NAME) or both throughout the experiment. In Pg group, left ventricular developed pressure, rate of rise in left ventricular pressure (dp/dt max), and rate pressure product were 83%, 55%, and 127%, respectively, higher than those of the control group ($p < 0.05$). The infarct size declined to less than 40% ($p < 0.0001$), and biomarkers of myocardial damage including creatine kinase-MB, lactate dehydrogenase, and troponin-I, showed significant reductions (59%, 36%, and 94%, respectively) compared with the control. Furthermore, the indices of oxidative status including superoxide dismutase, glutathione peroxidase, catalase, and malondialdehyde showed significant improvement (2.4, 1.7, 1.9, and 2.4 fold, respectively). Most of these effects were mainly blocked by L-NAME. These results suggest potent cardioprotective effects for Pg against myocardial I/R injury. The current results suggest a key role for NO for this cardioprotection; however, other mechanisms seem to be also involved.

KEYWORDS

ischemic heart, myocardial ischemia and reperfusion, nitric oxide, oxidative stress, pomegranate, *Punica granatum*

1 | INTRODUCTION

Myocardial ischemia and reperfusion (I/R) is the most prevalent cardiovascular disorder and the main cause of mortality worldwide (Mathers & Loncar, 2006). The formation of thrombosis on an atherosclerotic plaque in coronary arteries is the major cause of the disease that leads to an inadequate blood supply to the heart. In response to this failure, a number of molecular and cellular complexes are activated. The main aim of the intensive research in this area is to minimize the damage by intervening in these processes (Buja, 1998; Reimer & Ideker, 1987; Reimer & Jennings, 1992).

Plenty of mechanisms such as accumulation of intracellular Na^+, H^+, and Ca^{2+} ions (Buja, 2005); disturbed mitochondrial membrane potential leading to the formation of permeability transition pore (mPTP); apoptosis; and autophagy are involved in the pathogenesis of myocardial I/R injury (Turer & Hill, 2010). In addition, an increase in reactive oxygen species (ROS) during I/R causes an oxidant or antioxidant imbalance and is a key player in the etiology of the disease (Zweier, Flaherty, & Weisfeldt, 1987). Plant origin antioxidants have been widely used to ameliorate the damage via scavenging ROS (Koltai, Tosaki, Hosford, & Braquet, 1989; Varga et al., 1999). The endothelium-derived nitric oxide (NO), a key regulator of intracellular signaling pathways, is well known for its cardioprotective effects against I/R injury. The protective effects of NO are mediated via activation of K_{ATP} channels, inhibition of mPTP formation, modulation of electron transfer chain, production of cGMP, and antioxidants properties (Ferdinandy & Schulz, 2003; Maulik et al., 1995; Siu, Lotz, Ping, & Cai, 2015; Xi, Jarrett, Hess, & Kukreja, 1999).

There has been extensive research regarding the beneficial effects of plant-origin antioxidants on I/R injury. Using a working heart model, the isolated hearts from rats pretreated with standardized grape extract showed significant resistance to I/R injury (Cui, Juhasz, Tosaki, Maulik, & Das, 2002). In a similar study, the kernel extract of sour cherry (*Prunus cerasus*) caused a significant improvement in ventricular function of isolated hearts (Bak et al., 2006; Czompa et al., 2014). Resveratrol, a natural phenol found in red grapes, has shown similar cardioprotective effects in ischemic-reperfused hearts (Lekli et al., 2008; Mamunur Rahman, Bak, & Das, 2010).

Pomegranate fruit has been used as an herbal medicine in different countries (Langley, 2000). It has recently drawn great attention of scientific research. Pomegranate juice is a rich source of antioxidants such as ellagic tannin, gallotannins and anthocyanins, and flavonoids (Cerdá, Cerón, Tomás-Barberán, & Espín, 2003; Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). The juice demonstrates a significantly higher antioxidant activity compared with green tea and red grapes (Gil et al., 2000).

In addition to other therapeutic effects, anticarcinogenic (Panth, Manandhar, & Paudel, 2017), anti-inflammatory (Asgary, Keshvari, Sahebkar, & Hashemi, 2013), antinociceptive (González-Trujano, Pellicer, Mena, Moreno, & García-Viguera, 2015), and cardiovascular beneficial effects have been reported for the fruit (Davidson et al., 2009; Haseeb, Khan, Ashruf, & Haqqi, 2017; Razani, Dastani, & Kazerani, 2017). The juice has been reported to increase NO and prevent its destruction by ROS (Maulik et al., 1995; Siu et al., 2015; Xi et al., 1999). The current study aimed to evaluate the roles of nitric oxide and antioxidants in cardioprotection of pomegranate juice (Pg) against I/R injury.

2 | MATERIALS AND METHODS

The juice was prepared from the arils of the fruit using a manual pomegranate juicer. The juice was then lyophilized (Zirbus, Germany) and was stored under dry and dark conditions. The lyophilized powder was reconstituted with Krebs solution (1.8 mg ml^{-1}) and was then supplemented to the perfusion solution (2%: v/v), where applicable.

2.1 | Measurement of the total phenolic content

Total phenol content of the lyophilized juice was measured using Folin–Ciocalteu method (Singleton, Rossi, & Jr, 1965). Fifty milligrams of the sample was dissolved in 100-ml acidified methanol (HCl: 3%) and distilled water (60:40, v:v). The solution was filtered, and then 100 µl was mixed with an equal volume of Folin–Ciocalteu reagent (Sigma, USA) and 2 ml of sodium bicarbonate 2% solution. The samples were allowed to stay at room temperature for 2 hr, and then the optical absorbance was measured at 750 nm. The total phenol content was calculated according to gallic acid standard curve (0–10 mg ml^{-1}).

The main polyphenols of the juice were analysed using high-performance liquid chromatography-mass spectroscopy (Agilent Technology 6410-QQQ, USA and Japan). Chromatographic separation of the extract was carried out using a C18 column (250 × 4.6 mm; particle size: 5 µm, 25 °C). A gradient protocol was employed: Solution A contained 1% formic acid in distilled water, and Solution B was acetonitrile (flow rate: 0.5 ml min^{-1}). The percentage of Solution B was as follows: 0–5 min: 5%, 5–10 min: 10%, 10–15 min: 15%, 15–20 min: 20%, 20–25 min: 60%, and 25–30 min: 90%. The effluent of the column passed through the photodiode array detector (760 nm) and then was directed to the electrospray ionization interface and triple quadrupole mass spectrometer. A full scan mass spectrum (100–1,500 m/z) in negative-ion mode was employed for the electrospray ionization. The temperatures of the electrospray source was 100 °C. The capillary and cone voltages were 4.0 and 40 V, respectively. Nitrogen, as nebulising gas, was set at 25 psi (velocity: 6 L/min, 300 °C). The data processing was carried out using Agilent 6410 Triple Quad LC/MS Software. The main ingredients were quantified according to their peak area.

2.2 | The animals

Adult male Wistar rats (250–300 g) were obtained from the Animal House of the School of Veterinary Medicine, Ferdowsi University of Mashhad. All procedures were in accordance with the Guidelines of the Animal Welfare and Ethics Committee of the Ferdowsi University of Mashhad.

2.3 | Experimental groups

A randomized block design was used. The hearts were divided into four groups: (a) the control, (b) Pg 2%, (c) N^G-nitro-L-arginine methyl ester (L-NAME) 100 µM, and (d) Pg 2% plus L-NAME 100 µM. All the experimental groups experienced I/R. The test groups received Pg/LNAME via perfusion solution prior to global ischemia and throughout reperfusion.

2.4 | The Langendorff

The animals were anesthetized using thiopental sodium (50 mg k^{-1}, ip). Heparin (50 IU) was injected via dorsal penile vein. The chest was excised, and the heart was removed. It was immediately mounted on Langendorff setup and was perfused with modified Krebs solution containing (in mM) NaCl 118, $NaHCO_3$ 25, KCl 7.4, KH_2PO_4 2.1, $MgSO_4$ 1.2, $CaCl_2$ 25.1, and glucose 11 with a flow rate of 10 ml min^{-1} (pH: 7.4; 37 °C). Throughout the experiment, the solution was aerated with oxygen and carbon dioxide (95%:5%, v:v). Following 30-min stabilization, global ischemia was induced by cessation of Krebs solution inflow for 30 min. The hearts were then reperfused for 120 min.

During the experiment, hemodynamic parameters including heart rate (HR), coronary perfusion pressure (CPP), left ventricular developed pressure (LVDP), rate of rise in left ventricular pressure (dp/dt max), and rate pressure product (RPP) were monitored constantly using a PowerLab Electrophysiological Instrument (ML11, Australia). CPP was measured through a three-way stop cock just above the aortic cannula using a pressure transducer (MLT844 Physiological Pressure Transducer, ADInstruments). In order to measure LVDP, a small latex balloon, which was connected to another pressure transducer, was inserted into the left ventricle. Rate pressure product, an indicator

of the mechanical activity of the heart, was calculated as HR multiplied by LVDP.

2.5 | Biochemical parameters

The coronary effluent was sampled (10 ml) at specific time points for biochemical analysis. The samples were aliquoted upon collection and stored at −20 °C till assayed. Biochemical markers of myocardial I/R injury including creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were assayed using commercial spectrophotometric kits (Pars Azmun, Iran). The level of troponin I (TnI) in the effluent was measured using a chemiluminescence assay kit (Kate Vidas, Biomerieux, France). As an indicator of nitric oxide (Lee et al., 1999), the nitrite level of the coronary effluent collected at 2-min reperfusion was assayed using Griess method (Sib Biotec Co., Iran).

The heart homogenate was used for detection of the markers of oxidative stress. The superoxide dismutase (SOD) activity was measured using a Ransod assay kit (Randox, Germany). This method is based on the inhibitory effect of SOD on the generation of superoxide radicals due to oxidation of xanthine and subsequent formation of a red formazan dye (Arthur & Boyne, 1985; Suttle, 1986; Suttle & McMurray, 1983; Woolliams, Wiener, Anderson, & McMurray, 1983). The level of glutathione peroxidase (GPX) in the heart homogenate was measured based on the method of Paglia and Valentine (1967). Catalase (CAT) activity was determined based on the reaction of hydrogen peroxide with ammonium molybdenum. The resultant yellowish color was detected at 410 nm. The amount of malondialdehyde (MDA), an indicator of lipid peroxidation, in the homogenate was measured based on its reaction with thiobarbituric acid (Latha & Pari, 2003). The total protein of the heart samples was measured by biuret method (Pars Azmun, Iran).

2.6 | The infarct size

At the end of reperfusion, the hearts were dismounted from the aortic cannula, were rapidly frozen using liquid nitrogen, and stored at −20 °C. In order to measure the infarct size, the hearts were thawed and were cut into 2–3-mm transverse slices. The slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4; 37 °C) for 20 min and were then fixed in phosphate-buffered 4% formaldehyde for 24 hr. The slices were photographed, and the infarcted area was measured using Adobe Photoshop CS5 software (Andreka et al., 2007).

2.7 | Statistical analysis

GraphPad Prism Software V5 (GraphPad Software Inc, USA) was used for analysing the data and drawing the figures. Data are expressed as mean ± standard error. The hemodynamic, CK, and LDH were compared using two-way analysis of variance. All other comparisons were conducted using one-way analysis of variance followed by Bonferroni post hoc test. In all cases, $p < 0.05$ was considered as significant.

3 | RESULTS

3.1 | Chemical analysis of Pg

The total phenolic content of the juice was 4,200 mg/L. The main polyphenols including ellagic acid, gallagic acid, gallic acid, and punicalin were estimated to be 58.1%, 15.5%, 13.6%, and 12.8%, respectively (Figure 1).

3.2 | Hemodynamics of the hearts

The results for CPP, HR, dp/dt max, LVDP, and RPP in various experimental groups are shown in Table 1. Accordingly, prior to ischemia, there are no significant differences among different experimental groups. During ischemia, the parameters approached zero in all hearts. At the beginning of reperfusion, HR was significantly higher in Pg group compared with the control. The mean CCP was not statistically different among different groups. All other hemodynamic parameters, including LVDP, dp/dt max, and RPP were significantly higher in Pg group compared with other groups, both at the beginning and at the

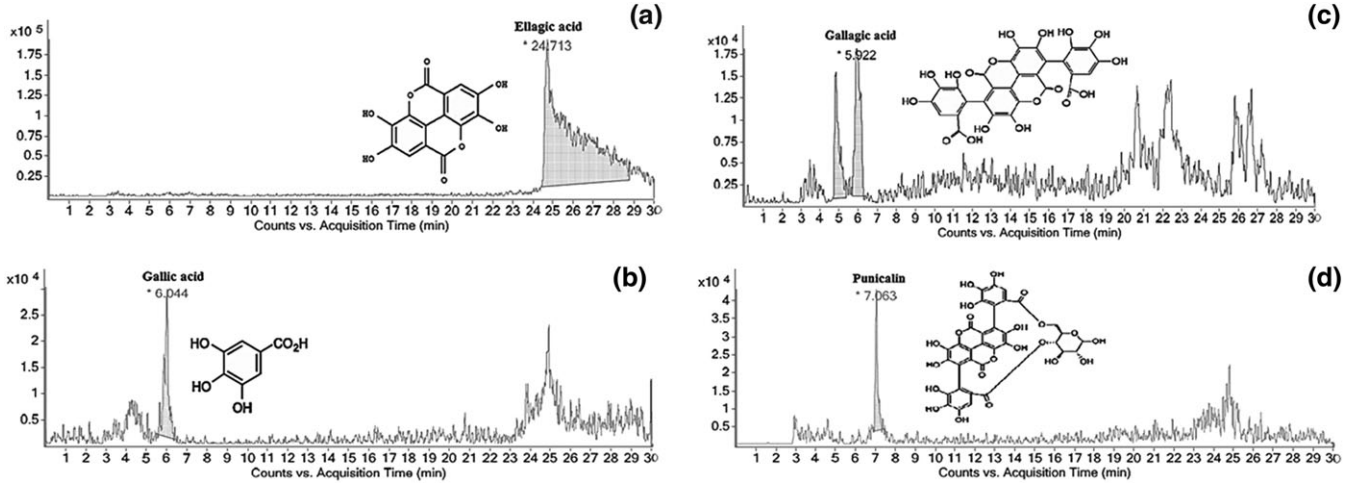

FIGURE 1 The liquid chromatography-mass spectroscopy chromatograph of pomegranate juice

TABLE 1 The effect of pomegranate juice on hemodynamics of the hearts following ischemia and reperfusion

		Stab	Ischemia			Reperfusion					
Time (min)		28	2	15	28	2	15	30	60	90	120
HR (beats/min)	C	247 ± 22.2	2 ± 2	0	0	144 ± 15.9	212.6 ± 23.7	239.1 ± 17.5	214.8 ± 19.9	220.9 ± 13.6	179.2 ± 14.7
	Pg	259.8 ± 12.8	4 ± 1.1	0	0	245.7 ± 17.2*	251.7 ± 13.5	251.7 ± 21.1	229.9 ± 19	245.1 ± 10.4	226.3 ± 9
	PgL	240.7 ± 10.3	2 ± 0.6	0	0	213 ± 24.6	239.4 ± 9.9	247.5 ± 20.9	230.3 ± 14.4	218.3 ± 12.7	235 ± 14.9
	L	255.4 ± 17.5	0	0	0	150.5 ± 10.2#	197.5 ± 13.4	196.2 ± 14.5	209.7 ± 16.9	190 ± 11	171.4 ± 13.6
CPP (mmHg)	C	88.1 ± 1.6	1.3 ± 0.3	0	0	82.6 ± 1.9	76.3 ± 2.1	75.8 ± 2.3	76.3 ± 1.5	76.7 ± 1.9	75.7 ± 1.8
	Pg	86.3 ± 1.1	1.8 ± 0.3	0	0	80.5 ± 1.1	75.9 ± 1.4	77.4 ± 2.1	73.5 ± 2.2	75.2 ± 1.9	72.8 ± 1.7
	PgL	86.5 ± 1.4	1.7 ± 0.5	0	0	81.5 ± 2.1	78.1 ± 1.6	78.3 ± 2.7	77.6 ± 2.2	75.9 ± 1.7	73.2 ± 1.2
	L	91.3 ± 1.7	1.3 ± 0.3	0	0	85.1 ± 1.5	78.7 ± 2.6	79.3 ± 3.1	78.1 ± 1.7	79.2 ± 1.5	78.4 ± 1.5
LVDP (mmHg)	C	34.1 ± 2.1	2 ± 0.4	0	0	27.8 ± 2.4	31.9 ± 4.3	31.2 ± 3.5	28.2 ± 2.2	24.4 ± 2.1	24.2 ± 1.4
	Pg	46.7 ± 4.3	3.9 ± 06	0	0	45.6 ± 3.6*	36.5 ± 4.9	35.5 ± 4.1	36.3 ± 2.5	30.5 ± 3.2	43.9 ± 3.7*
	PgL	38 ± 3.5	2.9 ± 0.9	0	0	30.1 ± 5.7#	30.8 ± 3	26.9 ± 1.3	29.5 ± 2.1	25.1 ± 2.8	26.2 ± 2.2#
	L	35.6 ± 4.2	1.9 ± 0.6	0	0	26.1 ± 3.3#	28.5 ± 3.5	25.6 ± 3.6	28.2 ± 4.2	27.1 ± 4	23.9 ± 4#
dp/dt max	C	1,065.7 ± 95	24.4 ± 0.8	26.2 ± 0.1	25.7 ± 0.5	781.9 ± 97.3	922 ± 106	959.5 ± 92.2	906.3 ± 81.4	881.7 ± 78.9	715.9 ± 90.8
	Pg	1,371.1 ± 65.7	23.1 ± 0.8	26.6 ± 0.1	26.6 ± 0.1	1,291.6 ± 64*	1,194.3 ± 77.9	1,214.8 ± 86.8	1,120.7 ± 85.1	1,036.1 ± 44	1,111.5 ± 86.8*
	PgL	1,059.7 ± 95.4	31.9 ± 4.1	25.6 ± 0.5	25.6 ± 0.3	920.9 ± 91#	997.3 ± 90.7	892.8 ± 103.3	983.3 ± 104.5	983.3 ± 104.5	753.6 ± 66.9#
	L	1,046.5 ± 54.3	24.9 ± 0.9	26 ± 0.3	25.5 ± 0.5	742.4 ± 89.1#	962 ± 94.1	932.3 ± 117.9	820.3 ± 84.2	802.4 ± 88.9	745 ± 67.3#
RPP	C	8,556 ± 945.1	3.3 ± 0.9	0	0	4,153.5 ± 762.9	7,070.6 ± 14..9	7,501.6 ± 1,098.3	6,133.7 ± 796.9	5,493.5 ± 690	4,328 ± 415.2
	Pg	11,777.9 ± 805.4	21.8 ± 7.6	0	0	11,223.5 ± 1,264.8*	9,203 ± 1,241.7	8,925.6 ± 1,369.5	7,989.6 ± 394.4	7,324.2 ± 757.5	9,842.2 ± 770.5*
	PgL	9,341.1 ± 1,111.9	5.2 ± 2.6	0	0	6,167.5 ± 988.9#	7,354.1 ± 830.3	6,795.9 ± 702	6,786.3 ± 658.3	5,498.1 ± 701.5	6,003.4 ± 517#
	L	9,061.2 ± 1,224.6	0.9 ± 0.5	0	0	4,023.6 ± 666.4#	5,714.4 ± 926.9	4,848.3 ± 762.4#	6,127.5 ± 1,231.9	5,161.4 ± 835.4	4,171.7 ± 808.2#

Note. Following 30-min stabilization (Stab), isolated rat hearts, in groups of 10, were subjected to 30-min global ischemia and 120-min reperfusion. The perfusion solution was Krebs for the control group (C), but it was further supplemented with 2% pomegranate juice (Pg), Pg plus L-NAME (PgL), or L-NAME (L) in the test groups. Heart rate (HR), coronary perfusion pressure (CPP); rate of rise in left ventricular pressure (dp/dt max); left ventricular developed pressure (LVDP), and rate pressure product (RPP) were monitored throughout the experiment. Data are presented as mean ± standard error. The asterisk indicate significant difference compared with the control group. The hash symbol indicates statistical difference compared with Pg group.

TABLE 2 The effect of pomegranate juice on the release of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) from isolated rat hearts

		Stab	Reperfusion	
Time (min)		**28**	**2**	**120**
CK-MB (μg/L)				
	C	21.6 ± 1.88	74.6 ± 5.6	57.15 ± 3.1
	Pg	16.3 ± 0.95	33 ± 1.7*	21.8 ± 1.9*
	PgL	20.8 ± 1.8	46.2 ± 1.8*#	35.9 ± 2.71*#
	L	21 ± 1.3	122.7 ± 3.9*#+	70.9 ± 2.8*#+
LDH (μg/L)	C	124.9 ± 2	260.4 ± 7.2	223.7 ± 10.1
	Pg	117.5 ± 3.6	174.5 ± 6*	137.5 ± 6.2*
	PgL	126.2 ± 4.1	250.4 ± 5.9*#	189.6 ± 8.5*#
	L	141.5 ± 9.2	315.2 ± 10.2*#+	276.5 ± 14.7*#+

Note. The experimental groups (n = 10 each) consisted of the control (C), 2% pomegranate juice (Pg), 2% Pg plus L-NAME (PgL), and L-NAME (L). All hearts experienced 30-min stabilization (Stab), 30-min global ischemia, and 120-min reperfusion. All information are expressed as mean ± standard error. Statistical significance has been shown by * (compared with the control), # (compared with Pg), or + (compared with PgL).

end of reperfusion period. Other intergroup comparisons did not yield statistical significance.

3.3 | Biochemical parameters

To evaluate the extent of myocardial damage, the levels of LDH, CK-MB (Table 2), and TnI (Figure 2) in the coronary effluent were measured. At the end of the stabilization period, these biomarkers were not significantly different among the experimental groups. However, the hearts treated with Pg had significantly lower levels of LDH and CK-MB compared with the control group, both at the beginning and at the end of reperfusion ($p < 0.0001$). Both Pg and L-NAME had significant effects on these parameters. However, the interaction between the two variables was only significant at the beginning of reperfusion. The amounts of TnI in the coronary effluent of Pg and PgL groups were less than 9% that of the control group ($p < 0.0001$). This biomarker was not significantly different in L group compared with the control.

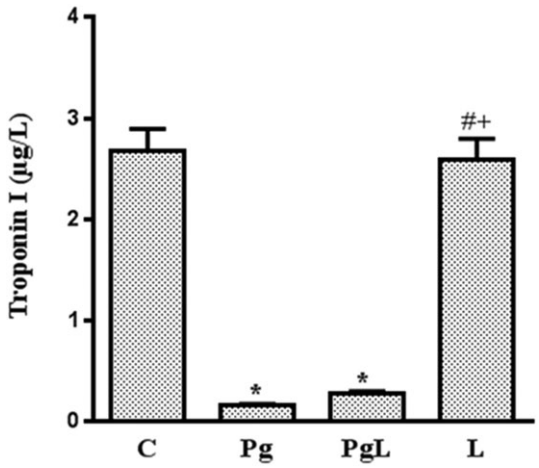

FIGURE 2 The effect of pomegranate juice on the release of troponin I following myocardial ischemia and reperfusion. The control group (C) was perfused with Krebs solution ($n = 10$), but the perfusion solution of other experimental groups ($n = 10$ each) was further supplemented with 2% pomegranate juice (Pg), Pg plus L-NAME (PgL), or L-NAME (L). Following 30-min stabilization (Stab), all heart were subjected to 30-min global ischemia and 120-min reperfusion. Data are shown as mean and standard error. The asterisk, hash, and plus symbols indicate significant differences compared with the control, Pg, or PgL groups, respectively

3.4 | Indices of oxidative stress

The biomarkers of oxidative stress including SOD, GPX, CAT, and MDA were assayed in different experimental groups (Figure 3). The level of SOD in Pg group was about 2.4 times that of the control group (1.32 vs. 0.54 U mg protein^{-1}; $p < 0.0001$). This parameter was significantly lower in PgL group (0.9 U mg protein^{-1}) compared with Pg group ($p < 0.001$). There was a significant difference between the control and L groups as well. The levels of GPX and CAT were significantly higher in Pg group compared with all other groups, and no statistical difference was observed among control, PgL, and L groups. The amount of MDA in Pg group was 42% that of the control group (4.04 vs. 9.62 nmol mg protein^{-1}; $p < 0.01$). This index was significantly higher in PgL group compared with Pg group, but L group was not statistically different from the control.

3.5 | Nitrite levels

The nitrite level, as an indicator of nitric oxide, was measured in the coronary effluent at the beginning of reperfusion (Figure 4). The amount of this chemical was remarkably higher in Pg group compared with the control (5.4 vs. 1.6 μM; $p < 0.01$). This parameter was significantly lower in PgL group. The nitrite level in L group was below that of the control ($p < 0.05$).

3.6 | The infarct size

The extent of the infarcted area in the control group had an average of 51% (Figures 5, 6). The infarct size declined to 20% in Pg group ($p < 0.0001$). This parameter was estimated 33% in PgL ($p < 0.01$ compared with Pg group and $p < 0.0001$ compared with the control) and 59% in L groups ($p < 0.0001$ compared with the control).

4 | DISCUSSION

This study investigated the protective effect of Pg against I/R induced myocardial injury in isolated rat heart. In order to verify the

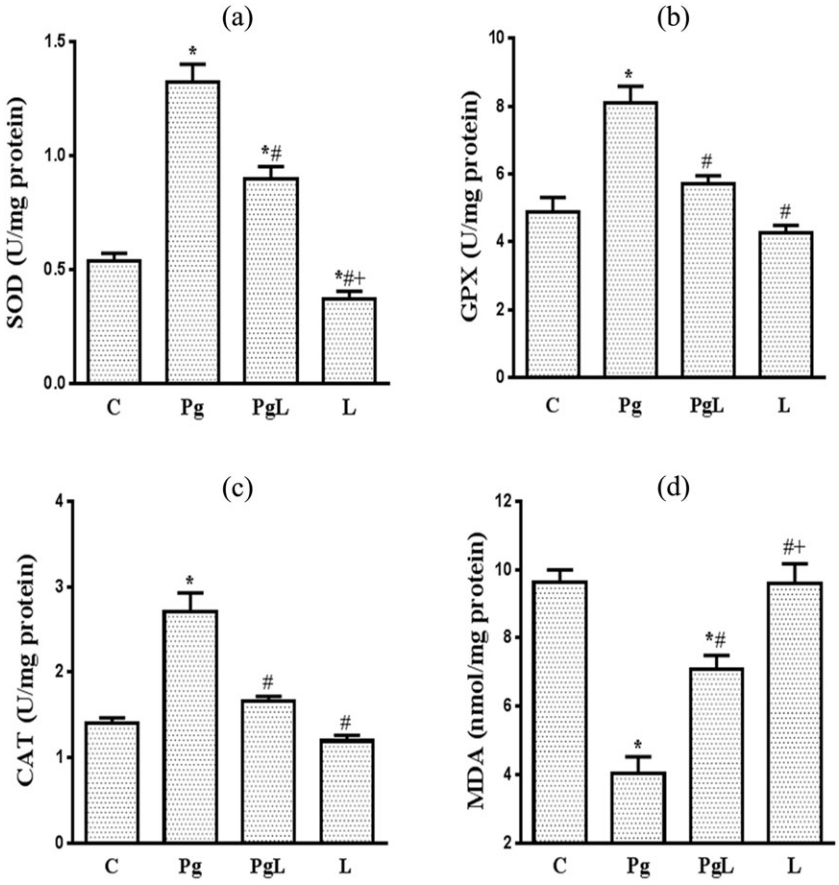

FIGURE 3 The biomarkers of oxidative stress following cardiac ischemia and reperfusion. The control hearts (C) were perfused with Krebs solution (n = 6), but the test groups (n = 6 each) further received 2% pomegranate juice (Pg), Pg and L-NAME (PgL), or L-NAME (L). The rat isolated hearts were subjected to global ischemia (30 min) followed by reperfusion (120 min). The levels of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and malondialdehyde (MDA) were measured in tissue homogenates. *: $p < 0.05$ versus control; #: $p < 0.05$ versus Pg; +: $p < 0.05$ versus PgL

involvement of NO in this cardioprotection, an inhibitor of NO synthase, L-NAME, was employed. The release of nitrite, as an indicator of NO, was also measured in the coronary effluent. Due to the existence of strong antioxidants in Pg, the markers of oxidative status were also studied in this research.

Pg improved the hemodynamic parameters upon reperfusion. This effect was abolished when L-NAME was added to the perfusion solution. This study suggested the involvement of NO in pomegranate-induced cardioprotection.

Myocardial cell death is one of the main outcomes of I/R injury. The extent of the damage was assessed via measurement of CK-MB,

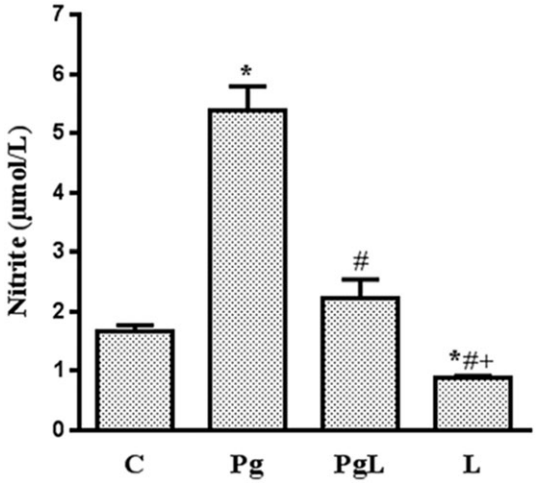

FIGURE 4 The effect of pomegranate juice on the release of nitric oxide from isolated hearts. All hearts underwent 30-min global ischemia and 120-min reperfusion. The test hearts (n = 6 each) were treated with 2% pomegranate juice (Pg), Pg and L-NAME (PgL), or L-NAME (L) and were compared with the control (n = 6). The data are represented as mean and standard error. The symbols *, #, and + indicate $p < 0.05$ compared with the control, Pg, and PgL groups, respectively

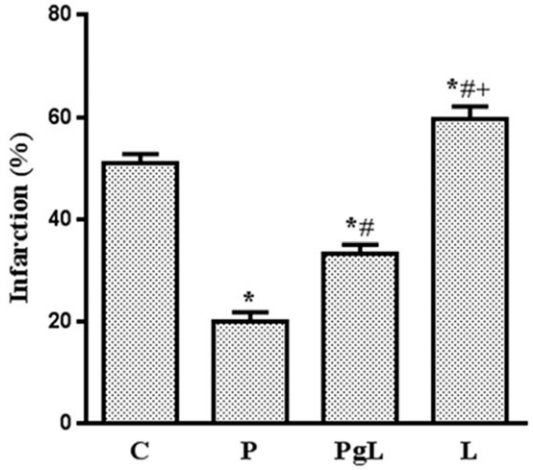

FIGURE 5 The effect of pomegranate juice on the extent of the infarcted area following myocardial ischemia (30 min) and reperfusion (120 min). The experimental groups (n = 10 each) consisted of the control (C), 2% pomegranate juice (Pg), Pg plus L-NAME (PgL), and L-NAME (L). All results are based on mean and standard error. Statistical significance compared with the control, Pg, and PgL groups are represented by *, #, and + symbols, respectively

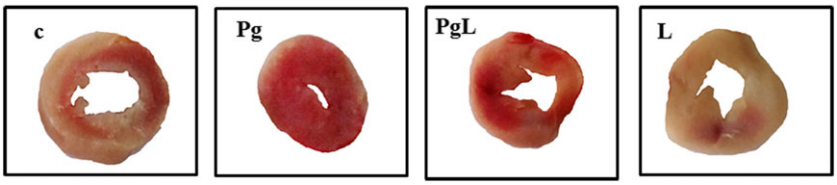

FIGURE 6 The cardiac slices in different experimental groups. The isolated perfused hearts from all experimental group were subjected to 30-min ischemia, followed by 120-min reperfusion. The test groups received pomegranate juice (2%: Pg), 3-NG-nitro-L-arginine methyl ester (L), or both (PgL) dissolved in the perfusion solution and were compared with the control group (C). The hearts were sliced and stained with 2,3,5-triphenyltetrazolium chloride for 30 min [Colour figure can be viewed at wileyonlinelibrary.com]

LDH, and TnI, as markers of cardiac cell damage, as well as TTC staining. The results suggest strong protective effects for the juice against I/R-induced cell death. Addition of L-NAME to the perfusion solution could attenuate, but not abolish, this effect, suggesting that NO is not alone in this process.

Reactive oxygen species play an important role in the pathogenesis of I/R-induced cell injury (Hearse & Tosaki, 1987a, 1987b; Zweier et al., 1987). Regarding the potent antioxidants such as polyphenols, flavonoids, and anthocyanins (Aviram & Rosenblat, 2012) in Pg, it was hypothesized that the juice could protect the heart against hypoxia and reoxygenation stress via scavenging ROS. Accordingly, the markers of oxidative stress including SOD, GPX, CAT, and MDA were analysed in the homogenates of the hearts. These markers suggest remarkable improvement in the oxidative status of the hearts in the presence of the juice. However, this effect was partially disappeared in the presence of the NO synthase inhibitor, L-NAME. This suggests that the increased level of NO due to Pg plays a partial role in the observed effect.

Pg possesses strong antioxidants ingredient such as polyphenols, flavonoids, and anthocyanins (Aviram et al., 2002). The juice has shown the highest polyphenol content and the most potent antioxidant activity compared with other studied fruit juices (Gil et al., 2000; Seeram et al., 2006). The juice has been shown to increase tissue level of the cardioprotective mediator, NO. In fact, the juice prevents the destruction of NO via neutralizing ROS (Ignarro, Byrns, Sumi, de Nigris, & Napoli, 2006). In addition, this procedure prevents the formation of the highly reactive and cytotoxic oxidant, peroxynitrite, thereby minimizes myocardial damage following I/R (Ferdinandy & Schulz, 2003; Liu, Zou, Slaughter, & Wang, 1997). L-NAME competes with L-argining, the natural substrate of nitric oxide, on the active site of eNOS, thus inhibiting the activity of the enzyme (Levick, 2009). According to the current results, L-NAME, per se, had minimal effects on the studied indices of oxidative stress. However, this NOS inhibitor significantly diminished the beneficial effects of Pg in this regard. The authors did not encounter another research regarding the impact of NO on the oxidative status of Pg-treated hearts. This is while the effects of nitric oxide on antioxidant enzymes of other organs are controversial (Dobashi, Pahan, Chahal, & Singh, 1997; Kostic, Andric, Maric, & Kovacevic, 2000; Rotzinger, Aragon, Rogan, Amir, & Amit, 1995). Apperantly, further research is needed to clarify the current findings.

There are few reports regarding the therapeutic effects of pomegranate fruit against myocardial I/R. This group had previously shown beneficial effects for the juice on the performance of isolated rat heart following global I/R (Rahimi, 2013). In an in vivo model of coronary occlusion and reperfusion in rat, pomegranate polyphenols caused significant reductions in LDH and CK-MB biomarkers (Dong, Tong, Liu, & Gao, 2012). In another study, pretreatment of rats with Pg for 30 days reduced the markers of cardiac damage and lipid peroxidation and attenuated the infarct size following isoproterenol-induced myocardial infarction (Jadeja, Thounaojam, Patel, Devkar, & Ramachandran, 2010). Consistent with the reports in animal studies, we showed in a clinical trial that 5 days of treatment with Pg caused a significant reduction in serum TnI of patients hospitalized for myocardial infarction (Razani et al., 2017).

The underlying mechanisms regarding the cardioprotective effect of Pg are not well understood. Regarding the potent antioxidant properties of the fruit, it is conceivable to attribute the therapeutic effect, at least partly, to destruction of ROS. During reperfusion, highly reactive oxidants are accumulated within cardiomyocytes. The resultant lipid peroxidation and oxidation of proteins leads to cell damage and death (Schmitt & Dirsch, 2009; Zweier et al., 1987). In this study, the markers of oxidative stress showed remarkable declines due to pomegranate treatment. Similar results have been reported by other researchers under in vivo conditions (Dong et al., 2012; Jadeja et al., 2010) including a clinical trial performed by our team (Razani et al., 2017). The current study, however, did not investigate the mechanism behind the increased NO levels. Accordingly, the possible effect of Pg on NO synthase activity should be also investigated.

Nitric oxide is an essential modulator of cardiovascular system. It plays a key role in the regulation of cardiac inotropy and chronotropy. Although inhibitors of constitutive NO do not interfere with cardiac function, NO donors may provoke negative inotropic and chronotropic responses in the heart (Han, Shimoni, & Giles, 1994; Hare & Colucci, 1995). In addition to these effects, NO is protective against myocardial I/R injury (Burwell & Brookes, 2008). In fact, NO improves heart function through several different mechanisms: First, nitric oxide increases the activity of K_{ATP} channels in both sarcolemmal and mitochondrial membranes of cardiomyocytes. As a result, the action potential and the duration of contraction is shortened. This means less need for adenosine triphosphate (ATP), and therefore, less cardiac damage (Gross & Auchampach, 1992; Qian, Levasseur, Yoshida, & Kukreja, 1996). Second, NO has an inhibitory effect on calcium overload in cardiac myocytes (Jones & Bolli, 2006). Third, NO preserves cellular homeostasis via inhibition of mitochondrial permeability transition

pore (Kim, Ohshima, Pediaditakis, & Lemasters, 2004). Finally, NO activates cyclooxygenase-2, which results in formation of cytoprotective prostanoids and leads to inhibition of proapoptotic proteins (Jones & Bolli, 2006).

Consistent to the current results, the beneficial role of NO following myocardial hypoxia and reperfusion has been reported by other researchers. Using an ex vivo model, the NO donor, S-nitroso-N-acetylpenicillamine, caused a significant reduction in infarct size and myocardial injury (Kanno et al., 2000). Similar effects were observed in an in vivo model when nitrite, a biological precursor of NO, was injected into the left ventricle prior to the induction of myocardial infarction (Duranski et al., 2005). In another study, pretreatment of rats with rosuvastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A-reductase, for 3 weeks, attenuated cardiomyocyte damage in excised hearts subjected to global I/R (Di Napoli et al., 2005). Consistent to these reports, the key role of NO in preconditioning and postconditioning is well documented (Jones & Bolli, 2006).

This study investigated the involvement of antioxidants and NO in cardioprotection caused by Pg following global I/R in isolated rat heart. The treatment significantly improved the markers of oxidative stress. The consequent rise in tissue level of NO had a pivotal role but could not fully justify the results. The antioxidative properties and the subsequent rise in NO levels seem to play crucial roles in cardioprotective effects of pomegranate.

CONFLICT OF INTEREST

There is no conflict of interest.

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REFERENCES

Andreka, G., Vertesaljai, M., Szantho, G., Font, G., Piroth, Z., Fontos, G., … Andreka, P. (2007). Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs. *Heart*, *93*, 749–752.

Arthur, J. R., & Boyne, R. (1985). Superoxide dismutase and glutathione peroxidase activities in neutrophils from selenium deficient and copper deficient cattle. *Life Sciences*, *36*, 1569–1575.

Asgary, S., Keshvari, M., Sahebkar, A., & Hashemi, M. (2013). Clinical investigation of the acute effects of pomegranate juice on blood pressure and endothelial function in hypertensive individuals. *ARYA Atheroscler*, *9*, 326–331.

Aviram, M., Dornfeld, L., Kaplan, M., Coleman, R., Gaitini, D., Nitecki, S., … Fuhrman, B. (2002). Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: Studies in atherosclerotic mice and in humans. *Drugs under Experimental and Clinical Research*, *28*, 49–62.

Aviram, M., & Rosenblat, M. (2012). Pomegranate protection against cardiovascular diseases. *Evidence-based Complementary and Alternative Medicine*, *2012*, 1–20.

Bak, I., Lekli, I., Juhasz, B., Nagy, N., Varga, E., Varadi, J., … Tosaki, A. (2006). Cardioprotective mechanisms of *Prunus cerasus* (sour cherry) seed extract against ischemia-reperfusion-induced damage in isolated rat hearts. *American Journal of Physiology Heart and Circulatory Physiology*, *291*, H1329–H1336.

Buja, L. (1998). Modulation of the myocardial response to ischemia. *Laboratory Investigation*, *78*, 1345–1373.

Buja, L. M. (2005). Myocardial ischemia and reperfusion injury. *Cardiovascular Pathology*, *14*, 170–175.

Burwell, L. S., & Brookes, P. S. (2008). Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. *Antioxidants & Redox Signaling*, *10*, 579–599.

Cerdá, B., Cerón, J. J., Tomás-Barberán, F. A., & Espín, J. C. (2003). Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *Journal of Agricultural and Food Chemistry*, *51*, 3493–3501.

Cui, J., Juhasz, B., Tosaki, A., Maulik, N., & Das, D. K. (2002). Cardioprotection with grapes. *Journal of Cardiovascular Pharmacology*, *40*, 762–769.

Czompa, A., Gyongyosi, A., Czegledi, A., Csepanyi, E., Bak, I., Haines, D. D., … Lekli, I. (2014). Cardioprotection afforded by sour cherry seed kernel: The role of heme oxygenase-1. *Journal of Cardiovascular Pharmacology*, *64*, 412–419.

Davidson, M. H., Maki, K. C., Dicklin, M. R., Feinstein, S. B., Witchger, M. S., Bell, M., … Aviram, M. (2009). Effects of consumption of pomegranate juice on carotid intima-media thickness in men and women at moderate risk for coronary heart disease. *The American Journal of Cardiology*, *104*, 936–942.

Di Napoli, P., Taccardi, A. A., Grilli, A., De Lutiis, M. A., Barsotti, A., Felaco, M., & De Caterina, R. (2005). Chronic treatment with rosuvastatin modulates nitric oxide synthase expression and reduces ischemia-reperfusion injury in rat hearts. *Cardiovascular Research*, *66*, 462–471.

Dobashi, K., Pahan, K., Chahal, A., & Singh, I. (1997). Modulation of endogenous antioxidant enzymes by nitric oxide in rat C6 glial cells. *Journal of Neurochemistry*, *68*, 1896–1903.

Dong, S., Tong, X., Liu, H., & Gao, Q. (2012). Protective effects of pomegranate polyphenols on cardiac function in rats with myocardial ischemia/reperfusion injury. *Nan Fang Yi Ke Da Xue Xue Bao*, *32*, 924–927.

Duranski, M. R., Greer, J. J. M., Dejam, A., Jaganmohan, S., Hogg, N., Langston, W., … Lefer, D. J. (2005). Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *The Journal of Clinical Investigation*, *115*, 1232–1240.

Ferdinandy, P., & Schulz, R. (2003). Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *British Journal of Pharmacology*, *138*, 532–543.

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry*, *48*, 4581–4589.

González-Trujano, M. E., Pellicer, F., Mena, P., Moreno, D. A., & García-Viguera, C. (2015). Antinociceptive and anti-inflammatory activities of a pomegranate (*Punica granatum* L.) extract rich in ellagitannins. *International Journal of Food Sciences and Nutrition*, *66*, 395–399.

Gross, G. J., & Auchampach, J. A. (1992). Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circulation Research*, *70*, 223–233.

Han, X., Shimoni, Y., & Giles, W. R. (1994). An obligatory role for nitric oxide in autonomic control of mammalian heart rate. *The Journal of Physiology*, *476*, 309–314.

Hare, J. M., & Colucci, W. S. (1995). Role of nitric oxide in the regulation of myocardial function. *Progress in Cardiovascular Diseases*, *38*, 155–166.

Haseeb, A., Khan, N. M., Ashruf, O. S., & Haqqi, T. M. (2017). A polyphenol-rich pomegranate fruit extract suppresses NF-κB and IL-6 expression by blocking the activation of IKKβ and NIK in primary human chondrocytes. *Phyther Res*, *31*, 778–782.

Hearse, D. J., & Tosaki, A. (1987a). Free radicals and reperfusion-induced arrhythmias: Protection by spin trap agent PBN in the rat heart. *Circulation Research*, *60*, 375–383.

Hearse, D. J., & Tosaki, A. (1987b). Reperfusion-induced arrhythmias and free radicals: Studies in the rat heart with DMPO. *Journal of Cardiovascular Pharmacology*, *9*, 641–650.

Ignarro, L. J., Byrns, R. E., Sumi, D., de Nigris, F., & Napoli, C. (2006). Pomegranate juice protects nitric oxide against oxidative destruction and enhances the biological actions of nitric oxide. *Nitric Oxide: Biology and Chemistry*, *15*, 93–102.

Jadeja, R. N., Thounaojam, M. C., Patel, D. K., Devkar, R. V., & Ramachandran, A. V. (2010). Pomegranate (*Punica granatum* L.) juice supplementation attenuates isoproterenol-induced cardiac necrosis in rats. *Cardiovascular Toxicology*, *10*, 174–180.

Jones, S. P., & Bolli, R. (2006). The ubiquitous role of nitric oxide in cardioprotection. *Journal of Molecular and Cellular Cardiology*, *40*, 16–23.

Kanno, S., Lee, P. C., Zhang, Y., Ho, C., Griffith, B. P., Shears, L. L. 2nd, & Billiar, T. R. (2000). Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. *Circulation*, *101*, 2742–2748.

Kim, J.-S., Ohshima, S., Pediaditakis, P., & Lemasters, J. J. (2004). Nitric oxide: A signaling molecule against mitochondrial permeability transition- and pH-dependent cell death after reperfusion. *Free Radical Biology & Medicine*, *37*, 1943–1950.

Koltai, M., Tosaki, A., Hosford, D., & Braquet, P. (1989). Ginkgolide B protects isolated hearts against arrhythmias induced by ischemia but not reperfusion. *European Journal of Pharmacology*, *164*, 293–302.

Kostic, T. S., Andric, S. A., Maric, D., & Kovacevic, R. Z. (2000). Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis. *The Journal of Steroid Biochemistry and Molecular Biology*, *75*, 299–306.

Langley, P. (2000). Why a pomegranate? *BMJ*, *321*, 1153–1154.

Latha, M., & Pari, L. (2003). Preventive effects of Cassia auriculata L. flowers on brain lipid peroxidation in rats treated with stretozocin. *Molecular and Cellular Biochemistry*, *243*, 23–28.

Lee, P. C., Salyapongse, A. N., Bragdon, G. A., Shears, L. L., Watkins, S. C., Edington, H. D., & Billiar, T. R. (1999). Impaired wound healing and angiogenesis in eNOS-deficient mice. *The American Journal of Physiology*, *277*, H1600–H1608.

Lekli, I., Szabo, G., Juhasz, B., Das, S., Das, M., Varga, E., … Tosaki, A. (2008). Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: The role of GLUT-4 and endothelin. *American Journal of Physiology Circulatory Physiology*, *294*, H859–H866.

Levick, J. R. (2009). *An introduction to cardiovascular physiology* (5th ed.). London: Springer.

Liu, X., Zou, H., Slaughter, C., & Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, *89*, 175–184.

Mamunur Rahman, M., Bak, I. K., & Das, D. (2010). Effectiveness of resveratrol against cardiovascular disease. *Mini-Reviews in Organic Chemistry*, *7*, 256–261.

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030 Samet J Ed. *PLoS Medicine*, *3*, e442.

Maulik, N., Engelman, D. T., Watanabe, M., Engelman, R. M., Maulik, G., Cordis, G. A., & Das, D. K. (1995). Nitric oxide signaling in ischemic heart. *CardiovascRes*, *30*, 593–601.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Panth, N., Manandhar, B., & Paudel, K. R. (2017). Anticancer activity of *Punica granatum* (pomegranate): A review. *Phytherapy Research*, *31*, 568–578.

Qian, Y. Z., Levasseur, J. E., Yoshida, K., & Kukreja, R. C. (1996). K_{ATP} channels in rat heart: Blockade of ischemic and acetylcholine-mediated preconditioning by glibenclamide. *American Journal of Physiology Circulatory Physiology*, *271*, H23–H28.

Rahimi K. (2013). Cardioprotective effect of pomegranate juice following myocardial ischemia and reperfusion. Sch Vet Med Ferdowsi Univ Mashhad, Mashhad.

Razani, Z., Dastani, M., & Kazerani, H. R. (2017). Cardioprotective effects of pomegranate (*Punica granatum*) juice in patients with ischemic heart disease. *Phytotherapy Research*, *31*, 1731–1738.

Reimer, K. A., & Ideker, R. E. (1987). Myocardial ischemia and infarction: Anatomic and biochemical substrates for ischemic cell death and ventricular arrhythmias. *Human Pathology*, *18*, 462–475.

Reimer, K. A., & Jennings, R. B. (1992). Myocardial ischemia, hypoxia, and infarction. *The Heart and Cardiovascular System*, 1875–1973.

Rotzinger, S., Aragon, C. M., Rogan, F., Amir, S., & Amit, Z. (1995). The nitric oxide synthase inhibitor NW-nitro-L-arginine methylester attenuates brain catalase activity in vitro. *Life Sciences*, *56*, 1321–1324.

Schmitt, C. A., & Dirsch, V. M. (2009). Modulation of endothelial nitric oxide by plant-derived products. *Nitric Oxide: Biology and Chemistry*, *21*, 77–91.

Seeram, N. P., Henning, S. M., Zhang, Y., Suchard, M., Li, Z., & Heber, D. (2006). Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours. *The Journal of Nutrition*, *136*, 2481–2485.

Singleton, V. L., Rossi, J. A., & Jr, J. (1965). Colorimetry of total phenolics with phosphomoybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*, 144–158.

Siu, K. L., Lotz, C., Ping, P., & Cai, H. (2015). Netrin-1 abrogates ischemia/reperfusion-induced cardiac mitochondrial dysfunction via nitric oxide-dependent attenuation of NOX4 activation and recoupling of NOS. *Journal of Molecular and Cellular Cardiology*, *78*, 174–185.

Suttle, N., & McMurray, C. (1983). Use of erythrocyte copper: Zinc superoxide dismutase activity and hair or fleece copper concentrations in the diagnosis of hypocuprosis in ruminants. *Research in Veterinary Science*, *35*, 47–52.

Suttle, N. F. (1986). Copper deficiency in ruminants; recent developments. *The Veterinary Record*, *119*, 519–522.

Turer, A. T., & Hill, J. A. (2010). Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American Journal of Cardiology*, *106*, 360–368.

Varga, E., Bodi, A., Ferdinandy, P., Droy-Lefaix, M. T., Blasig, I. E., & Tosaki, A. (1999). The protective effect of EGb 761 in isolated ischemic/reperfused rat hearts: A link between cardiac function and nitric oxide production. *Journal of Cardiovascular Pharmacology*, *34*, 711–717.

Woolliams, J. A., Wiener, G., Anderson, P. H., & McMurray, C. H. (1983). Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Research in Veterinary Science*, *34*, 253–256.

Xi, L., Jarrett, N. C., Hess, M. L., & Kukreja, R. C. (1999). Essential role of inducible nitric oxide synthase in monophosphoryl lipid a induced late cardioprotection: Evidence from pharmacological inhibition and gene knockout mice. *Circulation*, *99*, 2157–2163.

Zweier, J. L., Flaherty, J. T., & Weisfeldt, M. L. (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences of the United States of America*, *84*, 1404–1407.

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