Modulatory Effect of Nimbin on Isoproterenol Induced Mitochondrial and Lysosomal Enzymes Activities and Apoptosis Signaling in Rats

Yang WANG[1]*, Jingli CHEN[2]*, Haitao JIANG[3], Xiaoqin LI[4]

* These authors contributed equally to this work and are first co-authors.

[1]Department of Cardiovascular Medicine, First People's Hospital of Linping District, Hangzhou Zhejiang, China, [2]Renal Internal Medicine Dialysis Room, The Southwest Hospital of Army Medical University, Chongqing, China, [3]Department of Cardiovascular Medicine, Chifeng Municipal Hospital, Chifeng Inner Mongolia, China, [4]Department of Nephrology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiāngfan, Hubei, China

Summary

Nimbin, a bioactive triterpenoid compound isolated from the neem tree (*Azadirachta indica*), is known for its anti-inflammatory, antioxidant, antimicrobial, and hepatoprotective properties. The study aimed to explore the impact of nimbin on cardiac markers, mitochondrial and lysosomal enzyme activities, as well as apoptotic signaling in rats induced with isoproterenol. The subcutaneous injection of isoproterenol (ISO) at a dosage of 85 mg/kg body weight over the last two consecutive days led to notable increased in the activities/levels of the cardiac markers, lysosomal glycohydrolases and cathepsins. Conversely, reductions in the functioning of mitochondrial tricarboxylic acid cycle enzymes and respiratory chain enzymes in ISO-induced rats. In ISO-induced rats, there was an augmentation in the expressions of Bax, caspase-3, caspase-9, and cytochrome c, along with a diminished level of Bcl-2. Administration of nimbin resulted in decreased activities/levels cardiac markers, lysosomal glycohydrolases, cathepsins and increased functioning of mitochondrial tricarboxylic acid cycle enzymes and respiratory chain enzymes. Additionally, decreased expressions of Bax, executioner caspases and cytochrome c, along with heightened expression of Bcl-2, were noted in rats treated with nimbin. This indicates that nimbin possesses cardioprotective properties and mitigates mitochondrial and lysosomal dysfunction in rats induced with ISO.

Corresponding author

XQ. Li, Department of Nephrology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangfan Hubei, 441021, China. E-Mail: lxq1468054582@sina.com

Introduction

Cardiovascular disease (CVD) encompasses a range of conditions affecting the heart and blood vessels, including hypertension, atherosclerosis, heart failure, stroke, and congenital heart defects [1]. By 2030, it is forecasted that CVDs will emerge as the primary reason for mortality in India, with an estimated annual death toll of 23.3 million individuals [2]. It is noteworthy to highlight that myocardial infarction (MI) remains the primary contributor to cardiovascular diseases [3]. MI arises due to an elevated demand for myocardial metabolism and a reduced delivery of oxygen and nutrients to the myocardium through the coronary circulation, resulting in cellular damage [4,5].

In the present research, ISO, a catecholamine, was administered, serving as a standard model for investigating the positive impacts of various drugs on cardiac function. Previous studies suggest that subjecting the heart to increased catecholamine levels can lead to generation of necrotic tissue damage within the heart muscle of intervention animals [6]. Catecholamines experience quick self-oxidation, and there is a suggestion

that the oxidative derivatives of catecholamines play a role in the alterations observed in the myocardium. The ISO-induced myocardial necrosis resulted in modifications to membrane permeability, leading to the impairment of myocardial membrane function and integrity [7,8].

Mitochondria serve as the primary provider of cellular energy within our body. Various factors and substances have the potential to negatively impact the functioning of mitochondria. The leakage of lysosomal enzymes into the intracellular fluid is a significant factor in acute myocardial infarction [9]. Lysosomes are a group if cytoplasmic organelles present in numerous animal tissues that contain hydrolytic enzymes capable or degrading the cellular constituents. The release of these enzymes into the cytoplasm stimulates the formation of inflammatory mediators such as oxygen radicals and prostaglandins [10]. Lysosomal leakage can play an initiative role in apoptosis induced by oxidative stress. In myocardial ischemia, the increased activities of glycohydrolases usually decrease the lysosomal stability, resulting in necrosis of myocardium [11]. Decker et al. have reported that ISO-treated myocardial necrosis involves membrane permeability alterations that bring about loss of function and integrity of myocardial membranes [12].

Numerous medicinal plants are widely recognized for their ability to alleviate inflammatory conditions, including diabetes, infections, and cancer. Neem (*Azadirachta indica*) stands out as a medicinal plant with significant potential for addressing a variety of human health issues, including cardiovascular diseases. Neem has demonstrated its efficacy in treating various health disorders such as skin conditions, diabetes, dental and oral issues, as well as gastric ulcers, among others [13-17]. Nimbin sourced from neem, such as triterpenoids, and flavonols extracted from various sections of the botanical specimen, are attracting attention for their potential in addressing various diseases [18,19]. Nimbin, an active component of the neem tree, has been identified to possess diverse pharmacological activities. Mittal et al. conducted a study indicating that nimbin effectively interacted with the active sites of protein kinase B (Akt) and phosphatidylinositol 3-kinase (PI3K), exhibiting binding energies were found to be -8.2 and -6.8 kcal/mol, respectively [20]. According to Sharma et al. neem seed oil was identified to contain nimbin, which triggers apoptosis and halts the cell cycle in breast cancer [13]. Sharma et al. demonstrated that the combined encapsulation of doxorubicin and nimbin displayed heightened anticancer efficacy against THP-1 cells when subjected to near-infrared (NIR) light exposure [21]. Sudhakaran et al. reported that analogs of nimbin inhibit adipogenesis by suppressing reactive oxygen species (ROS) and superoxide anion while enhancing glutathione levels in zebrafish larvae exposed to a high-fat diet [22]. As per Sudhakaran et al. nimbin exhibited cytotoxic effects on MG-63 osteosarcoma cells, restraining cell proliferation and migration. It induced mitochondrial dysfunction, activated the caspase cascade, and stimulated apoptosis, ultimately resulting in programmed cell death [23].

The focus of this study was to examine the defensive properties of nimbin against isoproterenol-induced cardiac ischemia by assessing alterations in cellular respiratory organelles and intracellular degradation organelles in rat models. The study employed rat models to investigate nimbin's influence on myocardial mitochondrial and lysosomal enzymes when exposed to ISO-induced myocardial infarction. Additionally, we validated the impact of nimbin on apoptosis in a rat model induced with ISO.

Materials and Methods

Animal model

To assess the cardioprotective effect of nimbin, experiments were conducted using Swiss Albino male rats weighing 160-180 g, aged 5-7 weeks. The rats were obtained from Xiangyang Central Hospital and maintained in suitable conditions, receiving a standard diet with unrestricted access to drinking water. The research adhered to approved guidelines, and all parameters were scrutinized in compliance with the Institutional Animal Ethics Committee's recommendations.

Experimental induction of MI

To initiate myocardial infarction (MI), a dosage of 85 mg/kg body weight (b. wt.) of ISO was suspended in isotonic saline solution (0.9 % NaCl) and introduced *via* subcutaneous injection (s.c.) into the rat's right thigh. This process was repeated for two consecutive days, with a 24-hour interval between administrations.

Study design

The experimental setup involved four groups, each comprising six rats.

Group I – Control

Group II – Oral administration of nimbin (10 mg/kg b.wt.) for a period of 21 days

Group III – ISO control (85 mg/kg b.wt. at s.c.) for the last two consecutive days

Group IV – Oral administration of nimbin (10 mg/kg b.wt.) for a period of 21 days + ISO control (85 mg/kg b.wt. at s.c.) for the last two consecutive days.

The experiment concluded with the euthanasia of rats after an overnight fasting period. Rats were sedated through an intramuscular injection of ketamine. The blood drawn *via* cardiac puncture was collected and subsequently left to clot naturally at room temperature. The serum was prepared before being centrifuged at $400\times$ g, 10 min. The cardiac tissue was taken immediately, washed with saline and used for various analysis.

Serum biomarkers for MI evaluation

Serum samples were utilized to assess the enzymatic functions of CK-MB, cTnT, and cTnI. Commercial reagents obtained from Xiamen Boson Biotech Co., Ltd, Xiamen were employed for the measurement of CK-MB, cTnT, and cTnI activities, following the manufacturer's instructions.

Assessment of cardiac mitochondrial enzyme activity through tissue extraction

Mitochondrial-rich fractions were obtained from the heart tissue of various experimental animals using a method of differential centrifugation, as described in previously documented research. In summary, a homogenate with a concentration of 20 % (w/v) was formulated using a mixture of 0.25 M sucrose, 0.05 M Tris-HCl buffer, and 5.0 mM EDTA. The first centrifugation at $600\times$ g for 10 min efficiently separated cellular debris, tissue remnants, and cell nuclei, resulting in the formation of a nuclei pellet. Following this, the supernatant was subjected to centrifugation at $10000\times$ g for 5 min at 4 °C, resulting in the formation of the mitochondrial pellet. The remaining pellet, predominantly composed of healthy mitochondria, was carefully retained, while the upper part, potentially containing loose material, was discarded. The removal of lipid-rich foamy material was crucial to prevent uncoupling of mitochondria. Upon reconstitution of the mitochondrial pellet in a defined volume of potassium chloride (KCl), it was employed to determine multiple parameters, including the functionality of cardiac mitochondrial enzymes such as isocitrate dehydrogenase (ICDH) [24], succinate dehydrogenase [25], malate dehydrogenase (MDH) [26], α-ketoglutarate dehydrogenase (α-KGDH) [27], cytochrome c oxidase [28] and nicotinamide adenine dinucleotide dehydrogenase [29] were analyzed.

Isolation and assessment of lysosomal enzyme activity from cardiac tissue

The lysosomal fraction from the heart tissue was extracted following the procedure outlined by DePierre and Karnovsky [30]. Initially, fresh tissue was homogenized in ice-cold 0.25 M sucrose, followed by centrifugation at $3000\times$ g and $15000\times$ g. The resulting lysosomal pellet was resuspended in 1.15 % KCl for subsequent enzyme analysis, including β-glucuronidase [31], α and β-glucosidase [32], α and β-galactosidase [33], β-N-Acetyl glucosaminidase [34], Cathepsin-B [35], and Cathepsin-D [36] were analyzed.

Quantitative Real-Time RT-PCR in cardiac sample

Heart tissue-derived total RNA, extracted using TRIzol reagent, was quantified by measuring absorbance at 260 and 280 nm on a Biophotometer (Eppendorf). Subsequently, the RNA underwent reverse transcription into cDNA following established protocols. Real-time PCR was conducted in a StepOne Plus thermocycler (Applied Biosystems) using Power SYBR Green master mix, adhering to the manufacturer's instructions. Transcript expression levels of the target genes were standardized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The experiments were conducted independently three times, and the assessment of relative mRNA expression employed the $2^{-\Delta\Delta Ct}$ technique.

Western blot analysis

The heart tissues were washed with cold 1×PBS and then lysed for 30 min at 4 °C in RIPA buffer with protease inhibitor. Following lysis, the cell lysates were centrifuged for 10 min at 4 °C at $15000\times$ g. The protein samples (30 µg/lane) were loaded and electrophoresed on SDS-PAGE. By electroblotting, proteins were moved from the gel onto a nitrocellulose membrane. A 5 % bovine serum albumin-blocking solution was used to block the membrane protein for 2 h. Following blocking, TBST was used twice to wash the membrane. The membranes were incubated for 4 h at 4 °C with the 1st Ab (Bax, Caspase-3, -9, Cytochrome-c, Bcl-2 and

GAPDH) and washed twice with TBST and following probed with specific HRP-conjugated the 2nd Ab at 37 °C for 2 h. In the end, TBST was used to wash the membranes twice times. The protein bands were developed by chemiluminescent substrate.

Statistical analysis of the data

Statistical analyses were carried out using one-way analysis of variance (ANOVA), and comparisons between groups were performed using Duncan's Multiple Range Test (DMRT) *via* the SPSS/21 Software Package. The findings were reported as mean ± standard deviation for each group, with six rats in each group. Statistical significance was determined at a P-value below 0.05.

Ethical approval statement

The animal ethical approved by the Institutional Animal Ethics Committee's in the Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiāngfan, Hubei, China.

Results

Impact of nimbin on cardiac markers

ISO-induced groups showed that the elevation in serum concentration of CK, CK-MB, cTnT, cTnI and LDH. Nevertheless, pretreatment with nimbin restored all these cardiac indicators to baseline levels in ISO-induced rats (Table 1).

Evaluation of TCA enzyme response in ISO-induced rat hearts under the influence of nimbin

Mitochondrial dysfunction plays a significant role in the onset of various cardiovascular diseases (CVDs). To assess the effect of nimbin on tricarboxylic acid (TCA) cycle enzymes in rat heart tissue, we analyzed the activity of key enzymes crucial for mitochondrial function. Table 2 presents the mitochondrial enzyme activities in both the control and treatment groups. The enzymes involved in the oxidative metabolism of pyruvate in mitochondria – such as isocitrate dehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) – showed reduced activity in rats with myocardial infarction (MI) compared to the control group. However, rats pretreated with nimbin at a dose of 10 mg per kilogram of body weight exhibited a significant increase in enzyme activity compared to the ISO-induced group.

Effect of nimbin on lysosomal enzymes activities in control and ISO-induced rat hearts

Table 3 depict the performance of lysosomal enzymes in the hearts of control and ISO-induced rats. A marked increase was observed in the activities of β-glucosidase, α- and β-galactosidase, β-glucuronidase, cathepsin B, and cathepsin D in the cardiac muscle of rats with ISO-induced cardiac injury. However, in rats pretreated with nimbin, there was a significant reduction in the activity of these acid hydrolases in myocardial tissue compared to those treated with ISO alone.

Effect of nimbin on apoptotic gene expression in control and ISO-induced rats

To investigate the impact of nimbin on pro- and anti-apoptotic molecules in Figure 1. In heart tissue, there was a decline in Bcl-2 expression and an elevation in Bax, cytochrome c, caspase-9, and -3 expression in rats stimulated with ISO compared to reference rats. However, the application of nimbin to ISO-induced rats resulted in an upregulation of Bcl-2 expression and attenuation of Bax cytochrome c, caspase-9, and -3 expression when compared to ISO-induced rats.

Effect of nimbin on ISO-induced apoptotic protein expression in heart tissue

Cardiac apoptotic markers, including Bax, Bcl-2, caspase-3, -9 and cytochrome c were analyzed using Western blotting. In ISO-induced rats, the expression of these markers was significantly increased compared to control rats. However, treatment with nimbin markedly inhibited the expression of Bax, Bcl-2, caspase-3, -9 and cytochrome c in ISO-treated rats, as shown in Figure 2.

Discussion

Myocardial infarction poses a significant clinical challenge linked to cardiac damage, and the available therapeutic approaches are limited and often accompanied by notable side effects. Consequently, the effectiveness of various medications in providing cardio-protection is assessed using an experimental model induced by ISO to mimic myocardial infarction [37]. Many investigations have indicated that the physiological, structural, and metabolic shift observed in the hearts of intervention animals after the application of ISO closely resemble those seen in humans. The induced myocardial infarction by ISO stands as a standardized

Table 1. Effect of nimbin on cardiac markers in the serum of control and ISO-induced in experimental rats.

Groups	Control	Nimbin	ISO	Nimbin + ISO
CK (IU/l)	150.83 ± 17.92^a	149.47 ± 12.68^a	251.76 ± 20.41^b	181.27 ± 22.61^c
CK-MB (IU/l)	92.43 ± 8.37^a	90.28 ± 9.98^a	167.32 ± 17.19^b	121.27 ± 10.19^c
cTnT (ng/ml)	0.78 ± 0.07^a	0.78 ± 0.07^a	01.72 ± 0.19^b	01.10 ± 0.18^c
cTnI (ng/ml)	0.58 ± 0.06^a	0.56 ± 0.04^a	01.12 ± 0.14^b	0.71 ± 0.05^c
LDH (IU/l)	198.23 ± 18.05^a	197.78 ± 17.09^a	284.27 ± 28.22^b	219.17 ± 19.34^c

Data are expressed as means ± SD for six rats in each group. The data not sharing a common superscript (a, b, c) are different significantly at $p \le 0.05$ (DMRT). [a] No significant difference between control and Nimbin group. [b] Significantly different from the control group ($p<0.05$). [c] Significantly different from the ISO-induced group ($p<0.05$).

Table 2. Effect of nimbin on the activities of mitochondrial TCA cycle enzymes and respiratory chain enzymes in the heart of control and ISO-induced rats.

Parameters	Control	Nimbin	ISO	ISO + Nimbin
Mitochondrial TCA cycle enzymes				
ICDH (U#/mg protein)	785.15 ± 52.22^a	784.38 ± 62.18^a	358.27 ± 57.49^b	687.28 ± 61.08^a
α-KGDH (U*/mg protein)	88.32 ± 7.84^a	87.81 ± 7.93^a	46.41 ± 4.27^b	71.63 ± 6.84^c
SDH (U♠/mg protein)	206.82 ± 18.24^a	204.38 ± 10.19^a	164.76 ± 12.38^b	180.94 ± 16.83^c
MDH (U♦/mg protein)	344.81 ± 31.98^a	348.34 ± 4.56^a	226.47 ± 19.39^b	305.47 ± 29.19^c
Respiratory chain enzymes				
Cytochrome c oxidase (U♠/mg protein)	0.529 ± 0.108^a	0.404 ± 0.102^a	0.218 ± 0.118^b	0.447 ± 0.059^a
NADH dehydrogenase (U**/mg of protein)	128.24 ± 10.77^a	129.83 ± 10.21^a	75.29 ± 8.33^b	108.29 ± 8.73^a

Values are expressed as means ± S.D. for six rats in each group. Values not sharing a common superscript (a, b, c) differ significantly at $p<0.05$ (DMRT). U#=nmol of α-ketoglutarate liberated/hour, U*=nmol of ferrocyanide formed/hour, U♠=nmol of succinate oxidized/minute, U♠=nmol of NADH oxidized/minute, U♦=change in O.D/minute, U**=nmol of NADH oxidized/minute. [a] No significant difference between control and Nimbin group. [b] Significantly different from the control group ($p<0.05$). [c] Significantly different from the ISO-induced group ($p<0.05$).

Table 3. Effect of nimbin on the activities of lysosomal enzymes in the heart tissue of control and ISO-induced rats.

Parameters	Control	Nimbin	ISO	ISO + Nimbin
Heart				
α-Glucosidase (U♠/100 mg protein)	14.28 ± 1.53^a	14.58 ± 1.39^a	32.28 ± 4.66^b	18.83 ± 1.72^c
β-Glucosidase (U♠/100 mg protein)	8.17 ± 1.12^a	8.33 ± 0.88^a	21.29 ± 1.76^b	11.59 ± 1.17^c
α-Galactosidase (U♠/100 mg protein)	7.39 ± 0.48^a	7.41 ± 0.64^a	13.82 ± 1.15^b	9.27 ± 0.89^c
β-Galactosidase (U♠/100 mg protein)	15.41 ± 0.81^a	15.64 ± 1.43^a	29.27 ± 1.83^b	18.69 ± 1.93^c
β-Glucuronidase (U♠/100 mg protein)	9.29 ± 0.84^a	9.41 ± 0.89^a	18.37 ± 1.76^b	11.47 ± 1.04^c
β-N-Acetyl glucosaminidase (U♠/100 mg protein)	36.91 ± 2.72^a	38.37 ± 3.07^a	58.51 ± 5.05^b	40.47 ± 3.23^a
Cathepsin B (U*/100 mg protein)	22.14 ± 2.09^a	23.14 ± 2.73^a	44.73 ± 4.23^b	29.08 ± 2.06^c
Cathepsin D (U♦/100 mg protein)	17.29 ± 1.76^a	17.43 ± 1.52^a	27.62 ± 1.94^b	19.56 ± 1.79^c

Values are expressed as means ± S.D. for six rats in each group. Values not sharing a common superscript differ significantly at $p<0.05$ (DMRT). U♠=μmol of p-nitrophenol liberated/hour, U*=μmol of p-nitroaniline liberated/hour, U♦=μmol of tyrosine liberated/hour. [a] No significant difference between control and Nimbin group. [b] Significantly different from the control group ($p<0.05$). [c] Significantly different from the ISO-induced group ($p<0.05$).
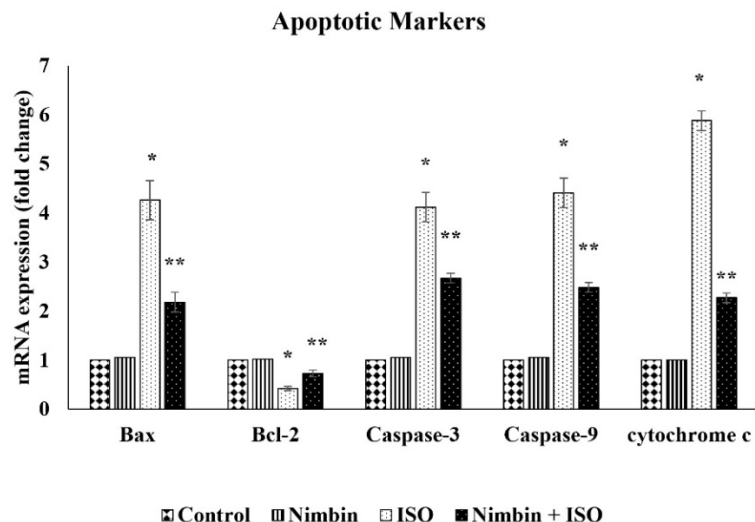
Apoptotic Markers

Fig. 1. Effect of nimbin on apoptotic gene expressions in heart tissue of control and ISO-induced experimental rats. The histogram depicts the quantitation of three independent experiments (means ± SD). The Bax, Bcl-2, caspase-3, -9 and cytochrome c genes expression levels were normalized with the expression level of the GAPDH mRNA in each sample. Values are expressed as means ± SD for three rats in each group. * Significantly different from the control group ($p < 0.05$). ** Significantly different from the ISO group ($p < 0.05$).
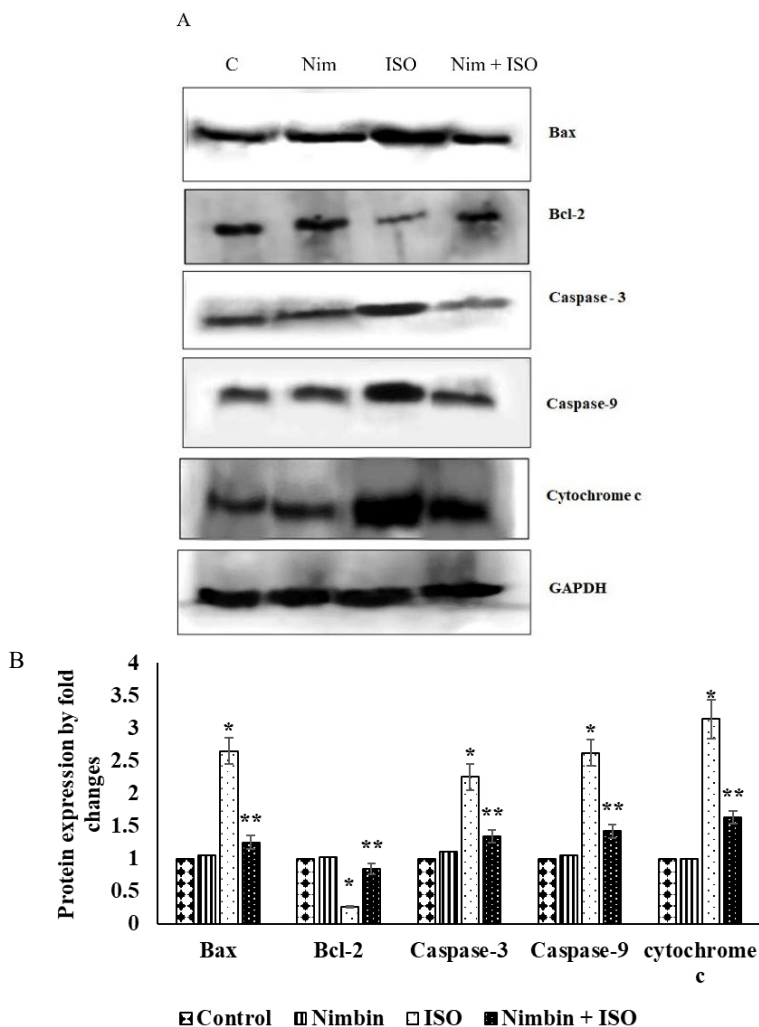

Fig. 2. Effect of nimbin on apoptotic protein expressions in heart tissue of control and ISO-induced experimental rats. (**A**) Effect of nimbin on ISO-induced Bax, Bcl-2, caspase-3, -9 and cytochrome c protein expression in heart tissue by Western blot analysis. (**B**) Densitometry analysis of apoptotic markers normalized by GAPDH. Values are expressed as means ± SD for three rats in each group. * Significantly different from the control group ($p < 0.05$). ** Significantly different from the ISO group ($p < 0.05$).

model for examining the positive impacts of various drugs on cardiac functions [38,39].

Studies have indicated that incorporating plant-based dietary agents can lower the susceptibility to cardiovascular diseases [40]. Nimbin, a natural compound derived from *Azadirachta indica*, has been traditionally utilized for the treatment of various ailments [41]. The focus of this research was to assess the cardioprotective effectiveness of nimbin against ISO-induced myocardial infarction by evaluating cardiac markers, as well as mitochondrial and lysosomal enzymes, along with examining apoptosis.

This investigation demonstrated a significant increase in myocardial markers, including CK-MB, cTn T, and cTn I, in the blood serum of ISO-infused rodents, in alignment with results documented by previous reported [42,43]. Under physiological circumstances, CK-MB is usually contained within cellular compartments, with its release into the bloodstream occurring following myocardial injury, as a result of the degradation of myofibrils and sarcotubular system [44]. Troponins T and I are precise polypeptide found in myocardial tissue, forming part of the troponin complex crucial for cardiomyocyte damage. These cardiomyocyte troponins serve as remarkable specific and discerning indicators of myocardial trauma, making them the ideal markers for diagnosing cardiac infarction [45,46]. The administration of nimbin led to a significant reduction in CK-MB activity and lowered the levels of cTnT and cTnI. This effect may be attributed to nimbin's ability to preserve membrane stability; this consequently limits the discharge of these cardiac markers into the bloodstream.

Mitochondria, vital subcellular structures responsible for energy generation, are susceptible to oxidative stress. Given their abundance in cardiomyocytes, these organelles play a crucial role in myocardial injury. Exploring the interactions between drugs and mitochondria as secondary targets can enhance our understanding of the underlying mechanisms. Mitochondrial dysfunction significantly impacts the pathogenesis of various cardiovascular diseases (CVDs). Within the myocardium, mitochondria occupy approximately 45 % of the volume of the heart muscle [47]. In ischemic conditions, the generation of lipid peroxides and hydrogen peroxide initiates cascading reactions that may harm mitochondrial membranes. Matsuzaki *et al.* observed that reactive oxygen species (ROS) generated during ischemia can harm various

macromolecules, including enzymes [48]. In our examination of ISO-induced rats, we noticed a reduction in the tasks of mitochondrial citric acid cycle enzymes (ICDH, SDH, MDH, and α-KGDH) within heart mitochondria. These enzymes, situated on the outer mitochondrial membrane, may be adversely impacted by the excessive generation of reactive oxygen species infused by ISO. Administration of nimbin to ISO-induced rats beforehand led to a notable increase in the activities of mitochondrial citric acid cycle enzymes. This enhancement could be attributed to nimbin's potential to hinder the production of reactive oxygen species and its capacity to scavenge them, as evidenced by our results.

The lysosomal vesicle membrane plays an indispensable role in regulating cellular protease secretion in pathophysiological conditions and inflammation-triggered situations. Rats treated with ISO exhibited elevated actions of acid hydrolases (β-glucuronidase, α and β-galactosidase, cathepsin-B and D) in both serum and cardiac muscle. The heightened lipid peroxidation seen in rats treated with ISO could potentially result in the liberation of blood and cardiac tissue proteases from intracellular vesicles as a consequence of lysosomal membrane impairment. It has been recorded that there were increased actions of serum and cardiac acid hydrolases in rodents treated with ISO [49]. Research has proven that the release of intracellular acid hydrolases from lysosomes and the muscle cell endoplasmic reticulum contributes to the malfunction and degradation of mitochondria, cell membrane, and additional cellular structures [2]. Premedication with nimbin normalized the functions of hydrolytic enzymes by inhibiting lipid peroxidation, thereby preventing ISO-induced damage to lysosomes in rats.

Disruption of the hydrolytic enzymes fidelity may result in an unwanted rise of enzyme levels both inside and outside the cell, potentially leading to cellular and tissue abnormalities, including apoptosis. Preserving the stability of the hydrolytic enzymes is essential for regulating standard levels of intracellular hydrolases and proteases in tissues and bodily fluids. The excretion of β-glucuronidase serves as an indicator of the structural integrity of the lysosomal barrier. The decrease in β-glucuronidase and cathepsin-D actions within the lysosomal component suggests a decline in membrane stability [50,51]. The heightened enzyme activities observed in the cytosolic portion stem from the discharge of these enzymes from the lysosomal compartment into the cytoplasm in rodents exposed to ISO treatment. Pre-

exposure with nimbin effectively hindered the dissemination of these enzymes from the intracellular compartment in ISO-treated rats. The redistribution of enzyme functions from the cytosolic compartment to lysosomal and cytosolic to overall enzymatic activities indicate a decrease in lysosomal stability in rats treated with ISO. The administration of nimbin prevented the excretion of acid hydrolases and reduced the overall function of intracellular hydrolytic enzymes thereby augmenting lysosomal stability. This outcome could be attributed to nimbin's membrane-stabilizing properties.

Existing literature proposes that oxidative stress-induced apoptosis of cardiomyocytes is a key factor in causing damage to cardiac tissue and advancing the progression of myocardial infarction [52]. Proteins such as Bcl-2 play a crucial role as regulatory components, safeguarding cells from cellular suicide, while Bax and caspase-3, identified as cell death-inducing genes, actively encourage cell demise. Certainly, redox imbalance triggers apoptosis networks by increasing the expression of Bax cellular factor and caspase protease, while concurrently reducing the levels of the antiapoptotic Bcl-2 [53,54]. This analysis reinforces the hypothesis by revealing a substantial rise in the regulation of apoptotic markers and cell death-associated genes and a diminished levels of anti-apoptotic factor – Bcl-2, following ISO treatment juxtaposed to the reference group. This occurrence has the potential to elevate apoptosis and lead to functional irregularities in the heart muscle. The halting caspase and Bax activation stands out as a significant strategy for mitigating myocardial apoptosis [55]. The administration of nimbin effectively restrained oxidative stress through its antioxidant properties. Additionally, nimbin treatment led to an elevation of Bcl-2 genes in the myocardial tissue, while simultaneously downregulating the expression of proapoptotic genes and apoptosis-related genes, Bax, caspase-3, -9 and cytochrome c in rats with ISO-stimulated myocardial infarction. This protective action helped safeguard myocardial cells from apoptosis.

Significance

The significance of this study demonstrates the cardioprotective effect of nimbin on myocardial infarction induced by ISO in rats. Nimbin's ability to protect heart tissue from damage caused by myocardial infarction, a condition where blood flow to the heart is severely reduced or blocked. Nimbin helps maintain the structural integrity of mitochondria and other intracellular organelles. Potential effect of nimbin to prevent apoptosis suggests it could help in preserving heart cells that would otherwise die in response to ischemic damage. This anti-apoptotic effect is crucial in mitigating the extent of myocardial injury and improving recovery outcomes. Overall, the study underscores nimbin's promising role in heart health, specifically in counteracting damage from myocardial infarction and supporting cellular repair and survival.

Conclusions

The study outcomes suggest that nimbin exhibits cardioprotective properties in rats with ISO-induced myocardial infarction by maintaining the structural integrity of both mitochondria and Intracellular digestive organelle membrane. Furthermore, nimbin restores enzyme activities to levels approximating normalcy in rats exposed to ISO-induced conditions. Additionally, nimbin demonstrates potential in preventing apoptosis, indicating its efficacy in averting apoptotic alterations. Hence, supplementation with nimbin appears advantageous in alleviating myocardial infarction induced by ISO.

Conflict of Interest
There is no conflict of interest.

Abbreviations
α-KGDH, alpha-ketoglutarate dehydrogenase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell leukemia/lymphoma-2; B.wt., Body weight; CK, creatine kinase; cTnI, cardiac troponin I; cTnT, cardiac troponin T; CVD, cardiovascular disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; ISO, isoproterenol hydrochloride; MDH, malate dehydrogenase; MI, myocardial infarction; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

References

1. Goldsborough E 3rd, Tasdighi E, Blaha MJ. Assessment of cardiovascular disease risk: a 2023 update. Curr Opin Lipidol 2023;34:162-173. https://doi.org/10.1097/MOL.0000000000000887

2. Akila P, Asaikumar L, Vennila L. Chlorogenic acid ameliorates isoproterenol-induced myocardial injury in rats by stabilizing mitochondrial and lysosomal enzymes. Biomed Pharmacother 2017;85:582-591. https://doi.org/10.1016/j.biopha.2016.11.067

3. Anderson JL, Morrow DA. Acute Myocardial Infarction. N Engl J Med 2017;376:2053-2064. https://doi.org/10.1056/NEJMra1606915

4. Thangaiyan R, Arjunan S, Govindasamy K, Khan HA, Alhomida AS, Prasad NR. Galangin Attenuates Isoproterenol-Induced Inflammation and Fibrosis in the Cardiac Tissue of Albino Wistar Rats. Front Pharmacol 2020;11:585163. https://doi.org/10.3389/fphar.2020.585163

5. Mohanty I, Arya DS, Dinda A, Talwar KK, Joshi S, Gupta SK. Mechanisms of cardioprotective effect of Withania somnifera in experimentally induced myocardial infarction. Basic Clin Pharmacol Toxicol 2004;94:184-190. https://doi.org/10.1111/j.1742-7843.2004.pto940405.x

6. Wong ZW, Thanikachalam PV, Ramamurthy S. Molecular understanding of the protective role of natural products on isoproterenol-induced myocardial infarction: A review. Biomed Pharmacother 2017;94:1145-1166. https://doi.org/10.1016/j.biopha.2017.08.009

7. Rathod S, Agrawal Y, Sherikar A, Nakhate KT, Patil CR, Nagoor Meeran MF, Ojha S, Goyal SN. Thymoquinone Produces Cardioprotective Effect in β-Receptor Stimulated Myocardial Infarcted Rats via Subsiding Oxidative Stress and Inflammation. Nutrients 2022;14:2742. https://doi.org/10.3390/nu14132742

8. Khalil MI, Ahmmed I, Ahmed R, Tanvir EM, Afroz R, Paul S, Gan SH, Alam N. Amelioration of Isoproterenol-Induced Oxidative Damage in Rat Myocardium by Withania somnifera Leaf Extract. Biomed Res Int 2015;2015:624159. https://doi.org/10.1155/2015/624159

9. Ferko M, Alanova P, Janko D, Opletalova B, Andelova N. Mitochondrial Peroxiredoxins and Monoamine Oxidase-A: Dynamic Regulators of ROS Signaling in Cardioprotection. Physiol Res 2024;73:887-900. https://doi.org/10.33549/physiolres.935513

10. Huang Q, Chen Y. NADPH oxidase 4 contributes to oxidative stress in a mouse model of myocardial infarction. Physiol Res 2023;72:177-186. https://doi.org/10.33549/physiolres.934992

11. Kumaran KS, Prince PS. Preventive effect of caffeic acid on lysosomal dysfunction in isoproterenol-induced myocardial infarcted rats. J Biochem Mol Toxicol 2010;24:115-122. https://doi.org/10.1002/jbt.20319

12. Decker RS, Poole AR, Griffin EE, Dingle JT, Wildenthal K. Altered distribution of lysosomal cathepsin D in ischemic myocardium. J Clin Invest 1977;59:911-921. https://doi.org/10.1172/JCI108713

13. Sharma R, Kaushik S, Shyam H, Agarwal S, Balapure AK. Neem Seed Oil Induces Apoptosis in MCF-7 and MDA MB-231 Human Breast Cancer Cells. Asian Pac J Cancer Prev 2017;18:2135-2140. https://doi.org/10.22034/APJCP.2017.18.8.2135

14. Charles V, Charles SX. The use and efficacy of Azadirachta indica ADR ('Neem') and Curcuma longa ('Turmeric') in scabies. A pilot study. Trop Geogr Med 1992;44:178-181.

15. Bandyopadhyay U, Biswas K, Sengupta A, Moitra P, Dutta P, Sarkar D, Debnath P, Ganguly CK, Banerjee RK. Clinical studies on the effect of Neem (Azadirachta indica) bark extract on gastric secretion and gastroduodenal ulcer. Life Sci 2004;75:2867-2878. https://doi.org/10.1016/j.lfs.2004.04.050

16. Bose A, Haque E, Baral R. Neem leaf preparation induces apoptosis of tumor cells by releasing cytotoxic cytokines from human peripheral blood mononuclear cells. Phytother Res 2007;21:914-920. https://doi.org/10.1002/ptr.2185

17. Kochhar A, Sharma N, Sachdeva R. Effect of supplementation of Tulsi (Ocimum sanctum) and Neem (Azadirachta indica) leaf powder on diabetic symptoms, anthropometric parameters and blood pressure of non-insulin dependent male diabetics. Studies Ethno Medicine 2009;3:5-9. https://doi.org/10.1080/09735070.2009.11886330

18. Paul R, Prasad M, Sah NK. Anticancer biology of Azadirachta indica L (neem): a mini review. Cancer Biol Ther 2011;12:467-476. https://doi.org/10.4161/cbt.12.6.16850

19. Nivetha R, Arvindh S, Baba AB, Gade DR, Gopal G, K C, Reddy KP, et al. Nimbolide, a Neem Limonoid, Inhibits Angiogenesis in Breast Cancer by Abrogating Aldose Reductase Mediated IGF-1/PI3K/Akt Signalling. Anticancer Agents Med Chem 2022;22:2619-2636. https://doi.org/10.2174/1871520622666220204115151

20. Mittal R, Chaudhry N, Mukherjee TK. Targeting breast cancer cell signaling molecules PI3K and Akt by phytochemicals Cannabidiol, Nimbin and Acetogenin: An in silico approach. J Biomed 2018;3:60-63. https://doi.org/10.7150/jbm.25815

21. Sharma V, Vijay J, Ganesh MR, Sundaramurthy A. Multilayer capsules encapsulating nimbin and doxorubicin for cancer chemo-photothermal therapy. Int J Pharm 2020;582:119350. https://doi.org/10.1016/j.ijpharm.2020.119350

22. Sudhakaran G, Rajesh R, Guru A, Arasu MV, Gopinath P, Arockiaraj J. Nimbin analogs N5 and N7 regulate the expression of lipid metabolic genes and inhibit lipid accumulation in high-fat diet-induced zebrafish larvae: An antihyperlipidemic study. Tissue Cell 2023;80:102000. https://doi.org/10.1016/j.tice.2022.102000

23. Sudhakaran G, Velayutham M, Aljarba NH, Al-Hazani TM, Arokiyaraj S, Guru A, Arockiaraj J. Nimbin (N1) and analog N3 from the neem seeds suppress the migration of osteosarcoma MG-63 cells and arrest the cells in a quiescent state mediated via activation of the caspase-modulated apoptotic pathway. Mol Biol Rep 2023;50:7357-7369. https://doi.org/10.1007/s11033-023-08627-7

24. Bell JL, Baron DN. A colorimetric method for determination of isocitrate dehydrogenase. Clinica Chimica Acta 1960;5:740-747. https://doi.org/10.1016/0009-8981(60)90017-6

25. Slater EC, Borner WD Jr. The effect of fluoride on the succinic oxidase system. Biochem J 1952;52:185-196. https://doi.org/10.1042/bj0520185

26. Mehler AH, Kornberg A, Grisolia S, Ochoa S. The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. J Biol Chem 1948;74:961-977. https://doi.org/10.1016/S0021-9258(18)57306-3

27. Steginsky CA, Gruys KJ, Frey PA. alpha-Ketoglutarate dehydrogenase complex of Escherichia coli. A hybrid complex containing pyruvate dehydrogenase subunits from pyruvate dehydrogenase complex. J Biol Chem 1985;260:13690-31369. https://doi.org/10.1016/S0021-9258(17)38780-X

28. Pearl W, Cascarano J, Zweifach BW. Microdetermination of cytochrome oxidase in rat tissues by the oxidation on N-phenyl-p-phenylene diamine or ascorbic acid. J Histochem Cytochem 1963;11:102-104. https://doi.org/10.1177/11.1.102

29. Minakami S, Ringler RL, Singer TP. Studies on the respiratory chain-linked dihydrodiphosphopyridine nucleotide dehydrogenase I. Assay of the enzyme in particulate and in soluble preparation. J Biol Chem 1962;237:569-576. https://doi.org/10.1016/S0021-9258(18)93963-3

30. DePierre JW, Karnovsky ML. Plasma membranes of mammalian cells: a review of methods for their characterization and isolation. J Cell Biol 1973;56:275-303. https://doi.org/10.1083/jcb.56.2.275

31. Van Hoof F, Hers HG. The abnormalities of lysosomal enzymes in mucopolysaccharidoses. Eur J Biochem 1968;7:34-44. https://doi.org/10.1111/j.1432-1033.1968.tb19570.x

32. Conchie J, Gelman AL, Levvy GA. Inhibition of glycosidases by aldonolactones of corresponding configuration. The C-4- and C-6-specificity of beta-glucosidase and beta-galactosidase. Biochem J 1967;103:609-615. https://doi.org/10.1042/bj1030609

33. Kawai Y, Anno K. Mucopolysaccharide-degrading enzymes from the liver of the squid, Ommastrephes sloani pacificus. I. Hyaluronidase. Biochim Biophys Acta 1971;242:428-436. https://doi.org/10.1016/0005-2744(71)90234-8

34. Moore JC, Morris JE. A simple automated colorimetric method for determination of N-acetyl-beta-D-glucosaminidase. Ann Clin Biochem 1982;19:157-159. https://doi.org/10.1177/000456328201900305

35. Barrett AJ. A new assay for cathepsin B1 and other thiol proteinases. Anal Biochem 1972;47:280-293. https://doi.org/10.1016/0003-2697(72)90302-8

36. Sapolsky AI, Altman RD, Howell DS. Cathepsin D activity in normal and osteoarthritic human cartilage. Fed Proc 1973;32:1489-1493.

37. Lobo Filho HG, Ferreira NL, Sousa RB, Carvalho ER, Lobo PL, Lobo Filho JG. Experimental model of myocardial infarction induced by isoproterenol in rats. (Article in English, Portuguese) Rev Bras Cir Cardiovasc 2011;26:469-476. https://doi.org/10.5935/1678-9741.20110024

38. Houston M. The role of nutrition and nutraceutical supplements in the treatment of hypertension. World J Cardiol 2014;6:38-66. https://doi.org/10.4330/wjc.v6.i2.38

39. Wang SB, Tian S, Yang F, Yang HG, Yang XY, Du GH. Cardioprotective effect of salvianolic acid A on isoproterenol-induced myocardial infarction in rats. Eur J Pharmacol 2009;615:125-132. https://doi.org/10.1016/j.ejphar.2009.04.061

40. Gan ZH, Cheong HC, Tu YK, Kuo PH. Association between Plant-Based Dietary Patterns and Risk of Cardiovascular Disease: A Systematic Review and Meta-Analysis of Prospective Cohort Studies. Nutrients 2021;13:3952. https://doi.org/10.3390/nu13113952

41. Gupta SC, Prasad S, Tyagi AK, Kunnumakkara AB, Aggarwal BB. Neem (Azadirachta indica): An indian traditional panacea with modern molecular basis. Phytomedicine 2017;34:14-20. https://doi.org/10.1016/j.phymed.2017.07.001

42. Chen W, Liang J, Fu Y, Jin Y, Yan R, Chi J, Liu W, Liu Y, Yin X. Cardioprotection of cortistatin against isoproterenol-induced myocardial injury in rats. Ann Transl Med 2020;8:309. https://doi.org/10.21037/atm.2020.02.93

43. Ouyang B, Li Z, Ji X, Huang J, Zhang H, Jiang C. The protective role of lutein on isoproterenol-induced cardiac failure rat model through improving cardiac morphology, antioxidant status via positively regulating Nrf2/HO-1 signalling pathway. Pharm Biol 2019;57:529-535. https://doi.org/10.1080/13880209.2019.1649436

44. Radhiga T, Rajamanickam C, Senthil S, Pugalendi KV. Effect of ursolic acid on cardiac marker enzymes, lipid profile and macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats. Food Chem Toxicol 2012;50:3971-3977. https://doi.org/10.1016/j.fct.2012.07.067

45. Park KC, Gaze DC, Collinson PO, Marber MS. Cardiac troponins: from myocardial infarction to chronic disease. Cardiovasc Res 2017;113:1708-1718. https://doi.org/10.1093/cvr/cvx183

46. Kumar M, Kasala ER, Bodduluru LN, Dahiya V, Lahkar M. Baicalein protects isoproterenol induced myocardial ischemic injury in male Wistar rats by mitigating oxidative stress and inflammation. Inflamm Res 2016;65:613-622. https://doi.org/10.1007/s00011-016-0944-z

47. Finsterer J, Ohnsorge P. Influence of mitochondrion-toxic agents on the cardiovascular system. Regul Toxicol Pharmacol 2013;67:434-445. https://doi.org/10.1016/j.yrtph.2013.09.002

48. Matsuzaki S, Szweda PA, Szweda LI, Humphries KM. Regulated production of free radicals by the mitochondrial electron transport chain: Cardiac ischemic preconditioning. Adv Drug Deliv Rev 2009;61:1324-1331. https://doi.org/10.1016/j.addr.2009.05.008

49. Radhiga T, Senthil S, Sundaresan A, Pugalendi KV. Ursolic acid modulates MMPs, collagen-I, α-SMA, and TGF-β expression in isoproterenol-induced myocardial infarction in rats. Hum Exp Toxicol 2019;38:785-793. https://doi.org/10.1177/0960327119842620

50. Bhat OM, Li PL. Lysosome Function in Cardiovascular Diseases. Cell Physiol Biochem 202;55:277-300. https://doi.org/10.33594/000000373

51. Chi C, Riching AS, Song K. Lysosomal Abnormalities in Cardiovascular Disease. Int J Mol Sci 2020;21:811. https://doi.org/10.3390/ijms21030811

52. Kurian GA, Rajagopal R, Vedantham S, Rajesh M. The Role of Oxidative Stress in Myocardial Ischemia and Reperfusion Injury and Remodeling: Revisited. Oxid Med Cell Longev 2016;2016:1656450. https://doi.org/10.1155/2016/1656450

53. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 2016;1863:2977-2992. https://doi.org/10.1016/j.bbamcr.2016.09.012

54. Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. Cell Death Differ 2018;25:65-80. https://doi.org/10.1038/cdd.2017.186

55. Chen Z, Wu J, Li S, Liu C, Ren Y. Inhibition of Myocardial Cell Apoptosis Is Important Mechanism for Ginsenoside in the Limitation of Myocardial Ischemia/Reperfusion Injury. Front Pharmacol 2022;13:806216. https://doi.org/10.3389/fphar.2022.806216