Cardioprotective effects of tilianin in rat myocardial ischemia-reperfusion injury

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Abstract. Tilianin, the main effective flavonoid monomer enriched from Dracocephalum moldavuca L., has been shown to have cardioprotective effects. However, the mechanism of tilianin cardioprotection remains largely unknown. The present study aimed to investigate the effects of tilianin preconditioning on myocardial ischemia/reperfusion injury and to analyze the possible mechanism of action. A total of 48 male Sprague Dawley rats were randomized into sham, model myocardial ischemia/reperfusion injury (MI/RI), propranolol hydrochloride positive control, and high-, medium- and low-dose tilianin groups (n=8 each). The rats in the tilianin groups were perfused with either 1.5, 2.5 or 5.0 mg/kg/d tilianin a week prior to surgery. The positive control group were perfused with 25 mg/kg/d propranolol. Saline was administered to the sham surgery and the MI/RI groups. The MI/RI model was established by ligating the left anterior descending coronary artery for 30 min, which was subsequently removed and the mice were observed for 120 min prior to sacrifice. Na+-K+-ATPase, Ca2+-ATPase, nitric oxide (NO), nitric oxide synthase (NOS) and endothelial system-related factors were analyzed using the respective detection kits. Immunohistochemistry was used to determine the expression levels of Bcl-2 and Bax. Caspase-3 activity was measured by quantitative polymerase chain reaction. The results showed that tilianin preconditioning significantly increased ATPase activity (P<0.01 and P<0.05) as compared with the model group (P<0.01 and P<0.05). Notably, the administration of tilianin significantly inhibited apoptosis, as evidenced by an increase in Bcl-2 expression, and reductions in Bax and caspase-3 mRNA expression levels (P<0.01 and P<0.05). These data indicate that pretreatment with tilianin exerts potent cardioprotective effects in rats with MI/RI. The anti-MI/RI effects comprised relieving calcium overload, correction of energy metabolism, improvement of endothelial function and inhibiting cell apoptosis.

Introduction

Cardiovascular disease is one of the most common life-threatening diseases, and it has been predicted that it will be the leading cause of mortality worldwide by 2030 (1). The predominant cause of mortality in cardiovascular disease is an acute myocardial infarction (AMI). Restoration of the blood flow to the ischemic myocardium is the most effective treatment principle of AMI. Therapeutic strategies, including thrombolysis and primary angioplasty (2), are frequently used in clinical practice. Nevertheless, reperfusion is considered to pose a risk, as it may result in a worsening of the tissue injury (3) and myocardial ischemia reperfusion injury (MI/RI), which may paradoxically reduce the beneficial effects of myocardial reperfusion, resulting in contractile dysfunction and cellular damage (4). The pathogenesis of MI/RI is complex and the mechanisms involved have not yet been fully elucidated. Previous studies have shown that active oxygen, calcium overload, neutrophil infiltration and apoptosis are all involved in the occurrence of MI/RI (5-9).

Dracocephalum moldavuca L. is a herb from the Labiatae family. This herb is traditionally used in Uyghur Medicine for the treatment of coronary heart disease, hypertension, atherosclerosis and myocardial ischemic disease in China (10). The predominant chemical constituents of Dracocephalum moldavuca L. are flavonoids, terpenoids, volatile oils, amino acids, trace elements and peptides. Tilianin is a predominant...
effective flavonoid monomer enriched from \textit{Dracocephalum moldavica L.} (11). As this traditional Chinese medicine monomer has a clear chemical structure and certain pharmacodynamic advantages, such as improved targeting, the utilization of Chinese medicine resources to analyze the efficacy of this compound is currently a key area of investigation. Nam et al (12) suggested that tilianin inhibits inducible nitric oxide synthase (iNOS) expression and production of nitric oxide (NO), and may act as an anti-inflammatory agent. Another previous study revealed tilianin to exert a protective effect in MI/RI (13), but the protective mechanism remains largely unknown. Therefore, further studies are required regarding the protective effects and mechanisms of tilianin on MI/RI.

The present study aimed to continue to investigate the cardioprotective effects of tilianin on MI/RI and elucidate its mechanism of action, in order to provide a theoretical basis for the clinical treatment of coronary heart disease with tilianin.

Materials and methods

**Drugs and reagents.** Tilianin was enriched from \textit{Dracocephalum moldavica L.} in the laboratory, and $^1$H-nuclear magnetic resonance (NMR), $^{13}$C-NMR, mass spectrometry and infrared spectrometry were used to identify the chemical structure. The purity obtained was $>$98%, as measured by high-performance liquid chromatography. The propranolol hydrochloride tablet (PHT) was purchased from Tianjin Lisheng pharmaceutical Co., Ltd (Tianjin, China) and sodium pentobarbital was supplied by Beijing Chemical Reagent Company (Beijing, China). The 2X Taq polymerase chain reaction (PCR) MasterMix was bought from Tiangen Biological Technology Co., Ltd (Beijing, China). The β-actin and caspase-3 primers were obtained from Bioneer Corporation (Daejeon, Korea).

**Animals.** The animal experiments carried out in the present study were approved by the Institutional Animal Care and Use Committee at Xinjiang Uyghur Autonomous Region Experimental Animal Research Center (Xinjiang, China). A total of 48 male Sprague Dawley rats, weighing 250-300 g, were provided by the Xinjiang Uyghur Autonomous Region Experimental Animal Research Center (certificate no.: XK 2003-0001; Xinjiang, China) and were housed under conditions of constant temperature and controlled illumination (light on between 8:30 and 20:30 h). Food and water were available \textit{ad libitum}.

**In vivo myocardial ischemia and reperfusion model.** All animals were assigned randomly to one of four groups: Sham group, rats were pretreated with saline, n=8; MI/RI group, rats were pretreated with saline, n=8; MI/RI + tilianin groups, high-, medium- and low-dose group rats were pretreated with tilianin (5.0 mg/kg, 2.5 mg/kg, 1.5 mg/kg respectively), n=24, 8 rats in each group; MI/RI + PHT group, rats were pretreated with PHT (25.0 mg/kg), n=8. Each treatment was orally administered for eight days, at a volume of 5 ml/kg weight once a day, and the surgical procedure was established 10 min after the last administration.

The rats were anesthetized with sodium pentobarbital (45 mg/kg injected intraperitoneally), and then intubated and artificially ventilated using a rodent ventilator (HX-100E; Chongqing Taimeng Technology Co., Ltd., Chongqing, China). A normal electrocardiograph was recorded following the placement of subcutaneous electrodes connected to an electrocardiograph (BL-420S; Chongqing Taimeng Technology Co., Ltd.). Ischemia/reperfusion was established as previously described by Zhao et al (14). Briefly, myocardial ischemia was induced by ligating the left anterior descending coronary artery (LAD) with a 3–0 silk suture. After 30 min ischemia, the ligature was released for 2 h, resulting in myocardial reperfusion (15,16). Sham-operated animals (sham group) underwent the same surgical procedures, with the exception that the 3-0 silk was passed around the left coronary artery but not tied. Successful myocardial ischemia was confirmed by ST segment elevation in the electrocardiogram, in addition to visual assessment of regional cyanosis of the ischemic region in the left ventricle. Reperfusion was confirmed by ST segment reversal and a color change in the ventricular surface from cyanotic to hyperemic (16). Arterial blood samples were collected at the end of the reperfusion, and the blood serum was separated at 1,200 x g and stored at 70°C. The mouse hearts were removed following the collection of blood and immediately placed in cold saline. The intracardiac blood was rinsed off and the left ventricle tissue was line-clipped under ligation and cryopreserved.

**Measurement of Na$^+$-K$^+$-ATPase and Ca$^{2+}$-ATPase activity.** Briefly, the frozen myocardial tissue was weighted and normal saline was added at a ratio of 1:9. Subsequently, the tissue homogenates were centrifuged to obtain the supernatant, followed by measurement of protein concentration using Coomassie Brilliant Blue kit (Nanjing Jiancheng Bioengineering Institute). Na$^+$-K$^+$-ATPase and Ca$^{2+}$-ATPase activity levels were determined by measuring the release of inorganic phosphate from ATP using the Na$^+$-K$^+$-ATPase kit and the Ca$^{2+}$-ATPase kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

**Measurement of NO and NOS activity.** NO is chemically reactive and can quickly convert to nitrate (NO$_3^-$) and nitrite (NO$_2^-$) \textit{in vivo}, with NO$_2^-$ further converting to NO$_3^-$ in the assay. NO$_2^-$ was reduced to NO$_3^-$ using nitrate reductase. The absorbance of NO$_3^-$ at 550 nm indirectly signifies the NO concentrations. NOS catalyzes L-Arg and molecular oxygen to produce NO and pronuclear material, which generates colored compounds. Thus, the absorbance at the 530 nm wavelength allows for calculation of the NOS activity. Using the serum and homogenate supernatant of each group, the NO levels and NOS activity were determined with a power wave XS2 enzyme-labeled instrument (Bio-Rad Laboratories, Hercules, CA, USA) using NO and NOS kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

**Measurement of endothelial system-related factors.** The serum levels of endothelin (ET)-1, calcitonin gene-related...
peptide (CGRP), thromboxane (TX) B₂ and 6-keto prostaglandin (PG) F₁α (6-Keto-PGF₁α) in the rat blood samples were measured using the corresponding rat ELISA kits (Shanghai Xitang Biological Technology Co., Ltd., Shanghai, China).

**Immunohistochemistry.** A section of ischemic tissue was fixed in 4% paraformaldehyde solution. After 24 h, this was dehydrated and then embedded in paraffin blocks. The blocks were then cut into 5 µm sections using a rotary microtome (Leica RM 2235; Leica Instrument Co., Ltd., Beijing, China).

Immunohistochemical procedures were conducted according to the manufacturer's instructions of the streptavidin-peroxidase kit (ZSGB-BIO, Beijing, China). Briefly, the tissue sections were dewaxed and dehydrated with xylene and alcohol. The sections were then incubated for 10 min with 3% H₂O₂ and an autoclave was used to heat the samples for 8 min for antigen retrieval. The samples were then incubated with the following primary antibodies: Bax (B-9; dilution 1:25; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and Bcl-2 (C-2; dilution 1:50; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Following three washes with phosphate-buffered saline, the sections were incubated with a non-biotin labeled goat anti-mouse IgG secondary antibody (ZSGB-BIO) for 30 min and further developed with 3,3-diaminobenzidine tetrahydrochloride (DAB). The sections were observed under an Olympus CX21 microscope (Xiamen Sannuxinu Electronic Technology Co., Ltd., Xiamen, China). The immunopositive cell rate was calculated as follows: Immunopositive cell rate = immunopositive cells/(immunopositive cells + immuno-negative cells) x 100%. The expression levels of the analyzed proteins were statistically compared using the staining intensity plus the percentage of positive cells. The score criteria are listed in Table I (17).

**Semi-quantitative (q) PCR analysis.** A centrifugal columnar RNAprep Pure Tissue kit (Tiangen Biological Technology Co., Ltd., Beijing, China) was used to extract the total RNA from the myocardial tissue. cDNA was first synthesized by reverse transcription PCR. An appropriate quantity of total RNA was used to synthesize cDNA using a reverse transcriptase kit (Thermo Fisher Scientific, Rockford, IL, USA) at the following conditions: 65˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min. Subsequently, this cDNA served as a template for the subsequent qPCR reaction: 94˚C for 3 min, 94˚C for 30 sec, 53˚C for 30 sec, 72˚C for 1 min and 72˚C for 35 cycles. The following primers were used in the reaction: β-actin forward, 5'-AGCCATGTACGTAGCCATCC-3' and reverse, 5'-CTCTCAGCTGTGGTGAA-3'; caspase-3 forward, 5'-TTGGAGCACTGTAGCACACA-3' and reverse, 5'-ACCACCTGAAGGATGGTAGCC-3'. The PCR products were detected by ionization on a 1.5% agarose gel.

**Statistical analysis.** Data are expressed as the means ± standard deviation. A Student's t-test was performed to analyze the differences between two groups. Data analyses were performed using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). A P<0.05 was considered to indicate a statistically significant difference.

<table>
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**Results**

**Effect of tilianin on Na⁺-K⁺-ATPase and Ca²⁺-ATPase activity.** The activity of Na⁺-K⁺- and Ca²⁺-ATPases in myocardial tissues was investigated in response to MI/RI. As compared with the sham group, Na⁺-K⁺- and Ca²⁺-ATPase activity was significantly reduced in the MI/RI group (Na⁺-K⁺-ATPase: sham, 11.97±1.21 versus MI/RI, 3.96±0.48, P<0.01; Ca²⁺-ATPase: sham, 3.66±0.28 versus MI/RI, 1.34±0.15, P<0.01). As
compared with the MI/RI group, significant recoveries in Na\(^+\)-K\(^-\)-ATPase and Ca\(^{2+}\)-ATPase activities were observed in the high- and medium-dose tilianin groups (P<0.01 and P<0.05 respectively), whereas no significant differences were observed between the low-dose tilianin group and the MI/RI group. The MI/RI + PHT group also exhibited a statistically significant increase in ATPase activity, in comparison with the MI/RI group (P<0.05; Fig. 1A).

**Effect of tilianin on NO and NOS activity.** As shown in Fig. 1B, serum NO levels and myocardial NOS activity were significantly increased following MI/RI surgery, as compared with the sham group [NO: sham, 21.38±2.66 versus MI/RI, 88.49±10.11, P<0.01; Total (t)NOS: sham, 19.87±2.08 versus MI/RI, 25.40±3.24, P<0.05; iNOS: sham, 14.88±0.51 versus MI/RI, 26.04±3.51, P<0.01]. In comparison with the MI/RI group, significant decreases (P<0.01 and P<0.05) were identified in the serum NO levels and myocardial iNOS activity upon administration of tilianin to MI/RI rats at the three dose levels analyzed. Only the high-dose tilianin group significantly reduced tNOS activity, as compared with the MI/RI group (P<0.01)

**Effect of tilianin on endothelial system-related factors.** The tilianin drug groups exhibited dose-dependent reductions in the levels of serum ET-1 and TXB\(_2\), and increases in the levels of serum CGRP and 6-Keto-PGF\(_{1α}\) as compared with the MI/RI group. The high- and medium-dose groups significantly differed from the model group (ET-1: MI/RI, 34.63±4.32 vs. MI/RI + high-dose tilianin, 21.64±2.82, P<0.01; CGRP: MI/RI, 1,479.58±235.29 vs. MI/RI + high-dose tilianin, 2,036.06±261.24, P<0.01; TXB\(_2\): MI/RI, 2,480.05±317.55 versus MI/RI + high-dose tilianin, 1,717.45±241.44, P<0.01; 6-Keto-PGF\(_{1α}\): MI/RI, 30.13±4.72 versus MI/RI + high-dose tilianin, 79.56±7.90, P<0.01). However, the MI/RI + PHT group exhibited reduced ET-1 and TXB\(_2\) levels and increased CGRP and 6-Keto-PGF\(_{1α}\) levels as compared with all the tilianin-treated groups. (Fig. 2)

**Effect of tilianin on Bcl-2 and Bax expression levels.** Bcl-2 protein was mainly accumulated in the cytoplasm. Immunohistochemical DAB coloration revealed diffuse distribution or brown granules in the cell cytoplasm. In the sham group, a few particles of Bcl-2 protein were detected (Fig. 3A). In the model group, positive staining of Bcl-2 protein was significantly increased in the cytoplasm (P<0.05). As compared with the model group, the tilianin drug groups exhibited significantly increased Bcl-2 protein expression levels (P<0.01 and P<0.05 for the high- and medium-dose groups, respectively; Fig. 3B).

The expression levels of Bax protein were similar to those of Bcl-2 protein, with an accumulation of Bax observed in the cytoplasm. Immunohistochemical staining revealed the sham group to exhibit almost no Bax protein expression (Fig. 3C); however, the Bax protein expression levels in the model group were significantly increased (P<0.01; Fig. 3D), as compared with those of the sham group. These data indicate that myocardial damage promotes apoptotic protein expression. Compared with the model group, the high- and medium-dose tilianin drug groups exhibited significantly decreased Bax protein expression levels (P<0.01). In addition, the high- and medium-dose tilianin drug groups exhibited significantly increased Bcl-2/Bax ratio, as compared with the model group (P<0.01; Fig. 3E).

**Effect of tilianin on caspase-3 mRNA expression levels.** Compared with the sham group, the expression levels of caspase-3 mRNA in the MI/RI group were significantly increased (sham, 0.37±0.37 versus MI/RI, 1.04±0.07, P<0.01). As compared with the MI/RI group, the tilianin high- and medium-dose groups exhibited significantly reduced caspase-3 mRNA expression levels (high-dose tilianin, 0.56±0.09, P<0.01; medium-dose tilianin, 0.88±0.06, P<0.05). In addition, the MI/RI + PHT group exhibited decreased expression levels of caspase-3 mRNA, as compared with those of the MI/RI group (MI/RI + PHT, 0.42±0.03, P<0.01). The results indicated that tilianin exerted a protective effect on the cardiovascular tissue that underwent MI/RI, by reducing the expression levels of caspase-3 mRNA (Fig. 4).

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Figure 2. Effect of Tilianin on endothelial system-related factors, measured by ELISA. (A) Serum levels of ET-1 and CGRP in each group. (B) Serum level of TXB\(_2\) and 6-Keto-PGF\(_{1α}\) in each group. **P<0.01 vs. Sham, *P<0.01, \(*P<0.05 vs.\ MI/RI, ET-1, endothelin 1; CGRP, calcitonin gene-related peptide, TXB\(_2\), thromboxane B2; 6-Keto-PGF\(_{1α}\), 6-keto prostaglandin F1α; MI/RI, myocardial ischemia/reperfusion injury; PHT, propranolol hydrochloride tablet.
Figure 3. Bcl-2 and Bax expression levels following MI/RI in each group of rats. (A) Expression levels of Bcl-2 protein in each group (magnification, x200). (B) Statistical analysis of Bcl-2 protein expression. (C) Expression levels of Bax protein in each group (magnification, x200). (D) Statistical analysis of Bax protein expression. (E) Statistical analysis of of Bcl-2/Bax (%). **P<0.01, *P<0.05 vs. Sham; **P<0.01, *P<0.05 vs. MI/RI. MI/RI, myocardial ischemia/reperfusion injury; PHT, propranolol hydrochloride tablet.

Figure 4. Caspase-3 mRNA expression levels in the myocardial tissue of each group. (A) Caspase-3 mRNA expression levels in each group. (B) Caspase-3/β-actin OD statistics. **P<0.01 vs. Sham, *P<0.01, *P<0.05 vs. MI/RI. OD, optical density; MI/RI, myocardial ischemia/reperfusion injury; PHT, propranolol hydrochloride tablet.
Discussion

The present study generated three notable findings: (i) Pretreatment with tilianin attenuated calcium overload and energy metabolism disorders in MI/RI, a condition that predominantly manifests through increased Na⁺-K⁺-ATPase and Ca⁺²-ATPase activity; (ii) reduced endothelial system-related factors, including NO and endothelial NOS, significantly contributed to the cardioprotective effect of tilianin; (iii) tilianin was demonstrated to protect against MI/RI by inhibiting apoptosis.

Traditional Chinese herbs are used worldwide and analyses of the effective components are being widely investigated. A number of studies (18,19) have confirmed that Dracunculaceae moldauca L. total flavonoid exerts a protective effect on MI/RI. Tilianin is an effective ingredient extracted from Dracunculaceae moldauca L. Previous studies have confirmed that the cardioprotective effects of tilianin in MI/RI are associated with reduced levels of intracellular enzymes that are released into the blood, attenuation of the overproduction of reactive oxygen species and reductions in the release of inflammatory factors, such as IL-1, IL-6 and tumor necrosis factor-α (13).

Na⁺-K⁺-ATPase is an integral membrane protein that maintains the normal physiological gradient across the cell membrane. This is achieved through coupling ATP hydrolysis to the transport of Na⁺ and K⁺. The Na⁺-K⁺-ATPase enzyme is comprised of two subunits, both of which have numerous isoforms and ion-pumping functions (20). For the transport of calcium ions, Ca⁺²-ATPase is the predominant active transport protein that can regulate intracellular calcium levels in numerous cell types. The Ca⁺²-ATPase has an important role in maintaining the cation gradient for homeostatic control of cellular properties, and is also crucial for the contractility and excitability of muscles (21,22). In the present study, Na⁺-K⁺-ATPase and Ca⁺²-ATPase activity was found to be reduced in the MI/RI group, but a significant recovery was observed in the tilianin drug group (Fig. 1). The results suggested that tilianin may protect the myocardium by increasing ATP enzyme activity, maintaining homeostasis of the intracellular environment and rectifying energy metabolism.

A number of studies have confirmed the cardioprotective effects of NO during MI/RI. In addition, the effects of NO on MI/RI that have been demonstrated include pro- and antiapoptotic effects, depending on the source of NO (23,24). However, the mechanisms underlying these effects are not completely understood. Previous studies have indicated that the beneficial effects of NO from NOS are mediated by the regulation of vascular tone, superoxide radical scavenging, and the inhibition of neutrophil adherence and infiltration (9,25). The results of the present study were contrary to those of previous studies, showing that myocardial NO expression levels and NOS activity was markedly increased following MI/RI injury. However, the rats of the tilianin drug-treatment groups exhibited significantly lower levels of NO and NOS (Fig. 1). These data indicate that tilianin may protect against MI/RI by reducing myocardial NOS activity and decreasing the production of NO, therefore reducing the cytotoxic effects of NO.

The endothelial system-related factors ET-1 and CGRP were investigated following tilianin administration. ET-1 is one of the most potent endogenous vasoconstrictor peptides so far identified (26); CGRP is a vasoactive peptide that exhibits a variety of physiological functions, including vasodilation (27). Together, ET-1 and CGRP are a pair of vasoconstriction and vasodilation factors that are mainly regulated through endothelial function. In addition, the dynamic balance of TXA₂/PGI₂ is the main regulatory system in maintaining angiogenesis and normal platelet function. However, these molecules are unstable in vivo due to a short half-life. Therefore, the concentrations of TXA₂ and PGI₂ are determined by monitoring the levels of the respective hydrolysis products, TXB₂ and 6-Keto-PGF₁α. In the tilianin drug groups, reduced serum ET-1 and TXB₂ levels, and increased serum CGRP and 6-Keto-PGF₁α levels were observed, as compared with the the MI/RI group. The results suggest that tilianin further protects against MI/RI by maintaining the balance of endothelial function-related factors.

Cardiomyocyte apoptosis is a predominant pathogenic mechanism underlying MI/RI injury (28). Apoptosis following ischaemia-reperfusion (I/R) has been determined to be associated with increased levels of Bax protein and a decreased Bcl-2/Bax ratio (29). The overexpression of Bcl-2 in mice has been shown to significantly inhibit apoptosis and decrease the infarct size in the mouse heart following I/R (30). Bax and Bcl2 are important mitochondrial regulators during cardiomyocyte apoptosis (31). Bcl-2 regulates the opening of mPTP in opposition to Bax, blocking cytochrome c release, inhibiting caspase activity and reducing cell apoptosis (32). Therefore, the roles of Bcl-2 and Bax proteins in the antiapoptotic effects of tilianin were investigated in the present study. The results showed that tilianin preconditioning significantly increased Bcl-2 expression levels and reduced Bax levels, with a corresponding increase in the Bcl-2/Bax ratio. The results indicated that changes in the ratio of proapoptotic to antiapoptotic proteins may also contribute to the antiapoptotic and cardioprotective effects of tilianin in I/R injury (Fig. 3).

Caspases are critical enzymes in the induction and execution of apoptosis, and can induce cellular destruction through the formation of apoptotic bodies. The caspase family has numerous members, and the effector caspase, caspase-3, is associated with the apoptotic cascade pathway (33), and is therefore widely used as an indicator of apoptosis. The results of the present study revealed that tilianin exhibited cardioprotective effects by inhibiting caspase-3 activity, and thus the activation of the cleavage process (Fig. 4).

In conclusion, these results demonstrate that tilianin exerts potent cardioprotective effects in rats with MI/RI. The effects of anti-MI/RI observed, included relief of calcium overload, correction of energy metabolism, improvement in endothelial function and inhibition of cell apoptosis. This suggests that tilianin is a potentially useful drug that may be applied clinically for the prevention or treatment of MI/RI.

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References