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The Effect of *Astragalus Mongholicus* Bunge Extract on Isoproterenol-Induced Myocardial Infarction in Rats

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Objectives: Our study's objective was to investigate the cardioprotective effects of *Astragalus mongholicus* Bunge, a herbal medicine cultivated in Mongolia. **Methods:** Fifty rats were divided into five groups of ten. The untreated control and treatment group rats received isoproterenol (150 mg/kg) by subcutaneous injection for two consecutive days on 29th and 30th days to induce myocardial infarction. The treatment groups received *Astragalus mongholicus* Bunge 71, 142, or 284 mg/kg for 28 days prior to infarction. At the end of the experiment, *Astragalus mongholicus* Bunge's cardioprotective effect was assessed through histopathology, the measurement of serum CK, AST, ALT, and LDH, and the oxidative stress markers malondialdehyde and total superoxide dismutase. **Results:** Treatment with *Astragalus mongholicus* Bunge 71 mg/kg caused significant decreases in the levels of LDH, AST, and CK serum compared with the control group ($p < 0.05$). Treatment with *Astragalus mongholicus* Bunge at 71, 142, and 284 mg/kg showed a significant decrease in malondialdehyde compared with the control group ($p < 0.05$). Treatment with *Astragalus mongholicus* Bunge at the same levels significantly reduced myocardial damage by protecting levels of the beneficial antioxidant enzyme total superoxide dismutase ($p < 0.05$). Histopathological examination of the group treated with *Astragalus mongholicus* Bunge 142 mg/kg showed a decreased area with necrosis and inflammatory cells in the myocardial tissue. **Conclusion:** The protective effect of *Astragalus mongholicus* Bunge occurs by protecting the myocardium's native superoxide dismutase levels, reducing the levels of malondialdehyde and serum marker enzymes LDH, AST, and CK.

Keywords: Myocardial Infarction, *Astragalus Mongholicus* Bunge, Isoproterenol, Cardiovascular Diseases

Introduction

In both developing and developed countries, cardiovascular disease (CVD) is considered the leading cause of morbidity and mortality. It has reached epidemic proportions due to the rapid socio-economic development in developing countries [1]. World Health Organization stated that in 2016 approximately 17.9 million people died from cardiovascular disease, representing 31% of all global deaths. Of these deaths, 85% are due to myocardial infarction (MI) and stroke [2]. CVD accounts for a significant proportion of the deaths of the world population. MI is an important acute disease of myocardial necrosis, manifested by an imbalance between myocardial blood demand and coronary blood delivery. This imbalance leads to cardiac ischemia and the degeneration of cardiomyocytes [3].

Isoproterenol (ISO) is a structurally synthetic catecholamine. ISO is a β-adrenoceptor agonist and can induce myocardial infarction (MI) at high doses. ISO-induced MI animals were used to show an excessive generation of free radicals followed by the production of oxidative stress due to reduced endogenous antioxidant activity [3, 4].

The genus *Astragalus* is in the Fabaceae family; there are about 2500 species distributed worldwide [5, 6]. *A. membranaceus, A. mongholicus* and *A. complanatus* have been mainly used in traditional medicine for their anti-inflammatory, immunostimulant, antioxidative, anticancer, antidiabetic, cardioprotective, hepatoprotective, anti-aging, and antiviral effects [7-10]. Polysaccharides, saponins, and flavonoids are the active constituents providing these effects [11]. Radix Astragali is an herbal medicine used worldwide as a medicinal and dietary supplement containing *Astragalus mongholicus* Bunge (AM) as an ingredient.

In 2001, Ambaga found that the use of *Astragalus variabilis* Bunge in combination with a selective blocker of cardiac beta-adrenergic receptors was effective in reducing the area of myocardial infarction in rats. The experiment was carried out on the rats after coronary arterial ligation. In the myocardial infarction region, there was the reversibly damaged border zone, which was partially saved from necrosis by using *Astragalus variabilis*. It moderately decreased the myocardial damage in the area of permanent and marginal ischemia [12]. In 1991, Avirmaa et al. found that the total polyphenolic compound of *Astragalus galactites* Bunge dilated the coronary arteries in cats with pituitrin-induced ischemic heart disease [13].

In traditional Chinese medicine, Astragalus is named Huáng Qí and this name has been widely used since the earlier period. The roots of this plant are considered to have a selective effect on heart function. According to Zhou in 2000, saponins, flavonoids, and astragalosides found in Astragalus membranaceus Bunge were shown to reduce MDA levels, increase heart muscle activity, and improve blood circulation in rats with myocardial ischemia-reperfusion [14]. According to the previous study's results, the species of Astragalus are capable of reducing the area of myocardial infarction by having antioxidant and anti-inflammatory activities resulting in lower serum marker enzymes. However, depending on the species, the medicinal plants' quality varied among different geographic locations [15]. Therefore, the purpose of this study was to investigate the cardioprotective effect of the roots of *Astragalus mongholicus* Bunge that was cultivated in Mongolia.

Materials and Methods

Plant material and extraction of *Astragalus mongholicus* Bunge

The *Astragalus mongholicus* root grown for four years in Kherlen Bayan-Ulaan, Delgerkhaan soum, Khentii provinces of Mongolia was used for the study. The plant species of *Astragalus mongholicus* Bunge was verified by the Plant Protection Research Institute of Mongolia (01/28/2019).

An extract of *Astragalus mongholicus* Bunge was prepared by mixing 100 g of the root's powder in 100 ml 40% ethanol creating a 1:1 liquid extract by the remaceration method. The liquid was evaporated using a vacuum evaporator. The remaining dry residue of 1:1 liquid extract was 27.78% of the original 100 g of root powder.

Reagent

Isoproterenol hydrochloride was purchased from Sigma Aldrich Co. (USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from MLBio Co. (China). Standards of astragaloside were obtained from MACKLIN (China). Standards of gallic acid were obtained from Sigma-Aldrich (USA). Folin-Ciocalteu reagent and aluminum chloride (AlCl3) from the Sangon Biotech Co. (China) were used.

Phytochemical analysis

Total triterpenoid saponin: Sample and standard (saponin) were treated with 400 µl vanillin-acetic acid reagent and 1.6 ml of perchloric acid. This reaction mixture was kept on a water bath at 70-75° for 15 min. It was then cooled on an ice bath for 2 min and ethyl acetate added to make the final volume 10 ml. Absorbance was taken at 550 nm after mixing it well. The total saponin content was expressed as mg Astragaloside equivalent (SE) per g of the plant sample.

Estimation of total flavonoid contents: A precisely weighed 1.0 g sample was extracted with 30 ml of 1% aqueous hydrochloric acid in ethanol and reflux for 30 min, and then filtered into a 50 ml volumetric flask. Two ml of the extract were put in a 25 ml volumetric flask and treated with 1 mL of 1% AlCl3. Ethanol was then added to make the final volume 25 ml, after which absorbance values were determined using a spectrophotometer (UNICO UV-2102 C, China) at 430 nm. The content of flavonoids in the extract was reported in quercetin equivalents [16].

Estimation of total polyphenolic compounds: The Folin-Ciocalteu method was used to determine the AM's total phenolic content. A 10.75% Na2CO3 solution with Folin-Ciocalteu reagent (dilution 1:10) was added to the extract. After 30 min, the absorbance value corresponding to total polyphenols was measured at 760 nm. The total polyphenolic content was measured as g/kg, and the calibration curve was established using gallic acid [17].

Animals

Fifty healthy male Wistar rats weighing 220 - 250g were provided from the animal house of the Research Center, Institute of Traditional Medicine and Technology of Mongolia. They were kept at 20 ± 1°C and 50 - 60% humidity, with a 12-hour light/dark cycle, with automatic ventilation 8 - 15 times every hour. The rats were fed a standard diet and could drink ad libitum.

Isoproterenol-induced myocardial infarction

The study investigated the protective role of *Astragalus mongholicus* Bunge's extract on ISO-induced MI in rats. Fifty rats were randomly divided into five groups: normal, control, AM 71 mg/kg, AM 142 mg/kg, and AM 284 mg/kg group with 10 rats in each group. The normal group's rats did not receive the AM extract or isoproterenol subcutaneous injection and were treated with oral saline were given once a day until the end of the experiment. The control group rats were given saline orally once a day for 28 days and then received isoproterenol (150 mg/kg body weight) subcutaneous injections on the 29th and 30th days. The three groups of rats treated with AM were treated with extract of *Astragalus mongholicus* Bunge orally at doses 71, 142, and 284 mg/kg respectively for 28 days, followed by isoproterenol on 29th and 30th days. Blood samples were collected on the 31st day to measure the cardiac enzymes creatine kinase (CK), aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) and the oxidative stress markers (malondialdehyde) MDA and total superoxide dismutase (SOD). The animals were sacrificed on the 31st day, and heart tissue for histopathology was obtained.

Biochemical estimation in serum

The serum was extracted from the blood via centrifugation at 3000 rpm (15 min at 30°C), and its CK, AST, ALT, and LDH levels were measured using a DIRUI DR7000D biochemistry analyzer (DIRUI Industrial, China).

Enzyme-linked immunosorbent assay (ELISA)

The blood was kept at room temperature for 15 minutes after collection, and the samples were centrifuged at 3000 rpm for 10 min to separate the serum. The level of MDA and SOD were measured by ELISA kits (Shanghai MLBIO Biotechnology Co. Ltd) according to the manufacturer's instruction using a microplate reader (ChroMate-4300, Awareness Technology Co., USA).

Histopathological examination

The heart tissue samples were fixed in 10% neutral formalin and dehydrated through a series of graded ethanol baths and embedded in paraffin wax. Then 2 - 4 µm thick sections were taken from the embedded tissue samples and stained with hematoxylin and eosin (HE). The histopathological examination was performed by an expert pathologist using light microscopy (Nikon Eclipse Ci) to detect pathological changes. The changes were graded using a semiquantitative scoring system.

Statistical analysis

The mean ± standard deviation (SD) were calculated for the observed values for each of the 5 experimental groups. The groups

were compared using a one-way ANOVA followed by a Tukey's post hoc test. Graphpad Prism-7 software was used for statistical analysis with $p < 0.05$ considered statistically significant.

Ethical statement

The study was approved by the Research Ethics Committee of the Mongolian National University of Medical Sciences (№2019/3-10).

Results

Total triterpenoid, saponin, polyphenolic and flavonoids contents

The content of total triterpenoid saponin in the AM extract was 8.65 ± 2.15%, reported as Astragaloside equivalent (standard curve equation: $y = 22.24 x - 0.0829$, $r^2 = 0.9777$). The AM extract's total polyphenol content was 0.37 ± 0.015%, reported as gallic acid equivalent (standard curve equation: $y = 110.77 x - 0.0736$, $r^2 = 0.995$). The extract's flavonoid content was 3.89 ± 0.163%, reported as quercetin equivalent (Table 1).

Effect of AM on activation cardiac marker enzymes of serum

The ISO-administered rats exhibited a significant rise in myocardial injury markers such as LDH, AST, and CK in blood serum compared with the normal group's rats ($p < 0.05$). The treatment with AM 71 mg/kg caused a significant reduction in the levels of LDH, AST, and CK in serum compared with the control group's rats ($p < 0.05$).

Table 1. Total triterpenoid saponin, polyphenolic and flavonoids in *Astragalus mongholicus* Bunge (AM)

Bioactive substance of AM	Standard reagent	Standard curve equation	Content (%) *
Triterpenoid saponin	Astragaloside	$y = 22.24 x - 0.0829$, $r^2 = 0.9777$	8.65 ± 2.15
Polyphenolic	Gallic acid	$y = 110.77 x - 0.0736$, $r^2 = 0.995$	0.37 ± 0.02
Flavonoids	Quercetin		3.89 ± 0.16

* mean ± SD

Table 2. Biochemical changes on *Astragalus mongholicus* Bunge (AM) in all experimental groups (mean ± SD)

	Groups				
	Normal	Control	AM 71 mg/kg	AM 142 mg/kg	AM 284 mg/kg
CK (U/L)	393.3 ± 87.63	835.0 ± 82.50#	475.0 ± 59.19*	582.7 ± 66.49**	596.4 ± 105.6Δ
AST (U/L)	165.5 ± 32.17	550.8 ± 135.23#	395.7 ± 27.66*	311.7 ± 98.18**	305.4 ± 53.93
ALT (U/L)	64.62 ± 13.77	96.14 ± 11.95	78.48 ± 16.76	81.94 ± 31.87	67.68 ± 20.90
LDH (U/L)	234.6 ± 34.09	847.3 ± 104.0#	547.0 ± 114.0*	688.2 ± 96.49	807.2 ± 97.83

AM = Astragalus mongholicus Bunge, CK = creatine kinase, AST = aspartate transaminase, ALT = alanine transaminase, LDH = lactate dehydrogenase. #Normal vs control group at $p < 0.05$; *Control vs AM 71 mg/kg group at $p < 0.05$; **Control vs AM 142 mg/kg group at $p < 0.05$; ΔControl vs AM 284 mg/kg group at $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test. n = 5-6 in each group.

Table 3. Antioxidant activity on *Astragalus mongholicus* Bunge in all experimental groups (mean ± SD)

	Groups				
	Normal	Control	AM 71 mg/kg	AM 142 mg/kg	AM 284 mg/kg
MDA (nmol/)	0.72 ± 0.1	1.59 ± 0.34#	0.97 ± 0.37*	1.03 ± 0.48**	0.95 ± 0.16Δ
SOD (pg/ml)	4.54 ± 0.53	2.63 ± 0.51#	5.35 ± 0.49*	6.09 ± 2.03**	5.74 ± 0.76Δ

AM = Astragalus mongholicus Bunge, MDA = malondialdehyde, SOD = total superoxide dismutase. #Normal vs control group at $p < 0.05$; *Control vs AM 71 mg/kg group at $p < 0.05$; **Control vs AM 142 mg/kg group at $p < 0.05$; ΔControl vs AM 284 mg/kg group at $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test. n = 6 in each group.

Table 4. Effect of *Astragalus mongholicus* Bunge on histopathological changes in the isoproterenol-induced myocardial infarction in rats

	Groups				
	Normal	Control	AM 71 mg/kg	AM 142 mg/kg	AM 284 mg/kg
Myonecrosis	-	+++	+++	++	++
Inflammation	-	+++	+++	++	+++

AM = *Astragalus mongholicus* Bunge. (+) Mild, (++) Moderate, (+++) Severe, (-) nil.

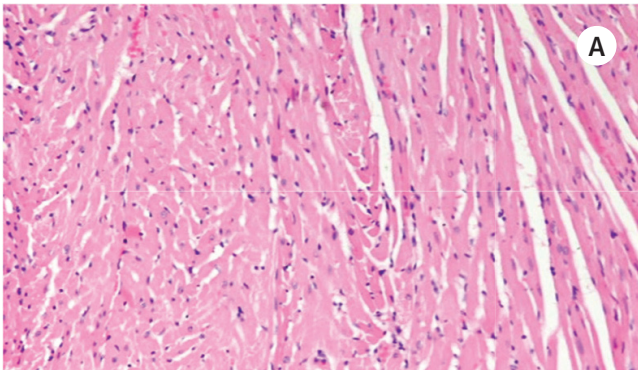
Furthermore, the rats treated with AM 142 and 284 mg/kg also exhibited a significant decrease in AST and CK compared with the control group ($p < 0.05$), while no significant alterations in ALT were observed in all the groups compared with the normal group's rats (Table 2).

Effect of AM on activation cardiac marker enzymes of serum

ISO administration in rats significantly increased the level of MDA (a marker of oxidative damage) in the heart compared with saline control rats ($p < 0.05$). However, AM 71, 142, and 284 mg/kg pretreatment produced a significant reduction in ISO-induced oxidative damage, as demonstrated by decreased levels of malondialdehyde (MDA) compared with saline control rats ($p < 0.05$). Furthermore, we observed that ISO markedly diminished the myocardial antioxidant enzyme SOD when myocardial damage in the normal group was compared with the saline control group ($p < 0.05$). Treatment with AM 71, 142, and 284 mg/kg significantly reduced the myocardial damage ($p < 0.05$) by protecting levels of the beneficial antioxidant enzyme SOD (Table 3).

Histopathological examination

Microscopically, there was necrosis of cardiomyocytes in all of the groups receiving ISO, as indicated by the disappearance of the cell nuclei, increased macrophages, few capillaries, and scar formation in the injured myocardium. Red blood cells, fibrin, neutrophils, and macrophages formed thrombus in the endocardium. Myocardial atrophy and fibrosis were observed as well. Macrophage and lymphocyte cell infiltration was seen in the myocardium and endocardium of all groups treated AM. Their necrotic myocardial cells had been removed and macrophages, connective tissue, and newly vascularization were seen in the endocardium. The AM 142 mg/kg treated group had fewer inflammatory cells in the myocardial tissue (Figure 1, Table 4).



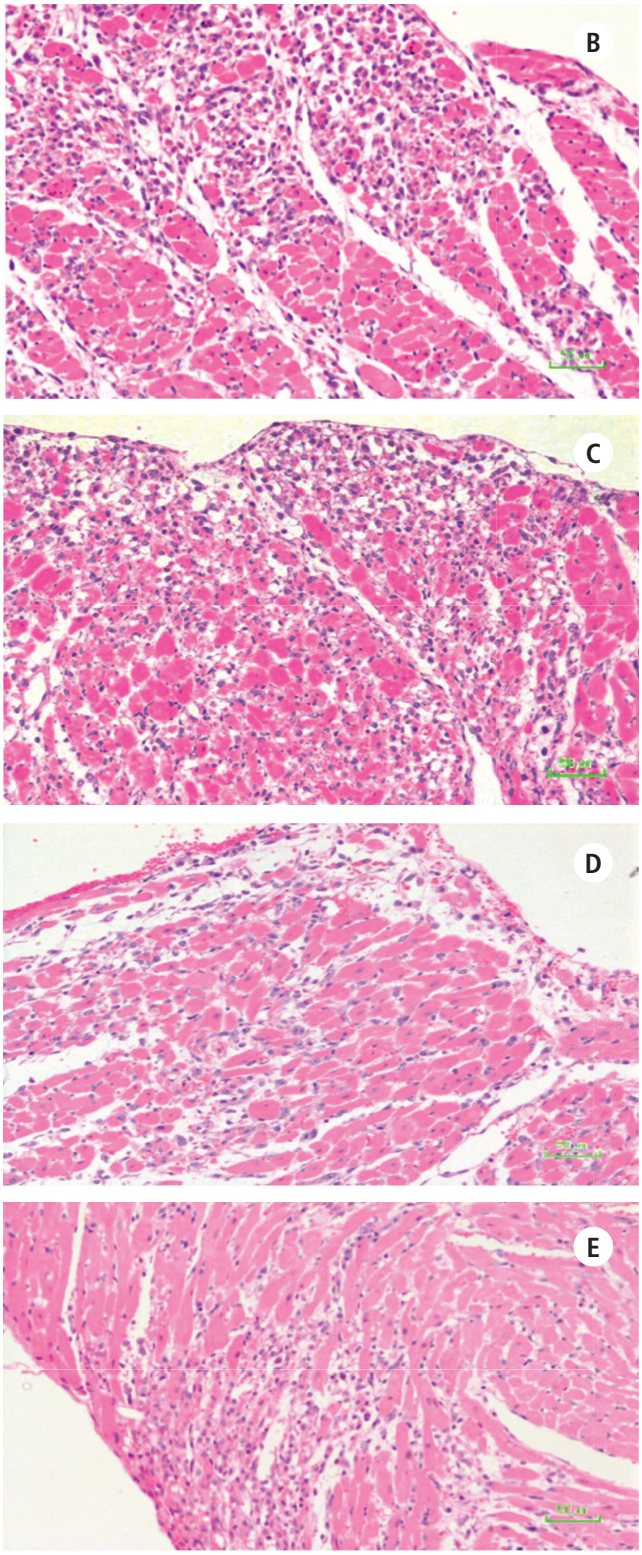


Figure 1. Histopathological changes in the heart tissue of normal, control and Astragalus mongholicus Bunge (AM)-treated groups. Hematoxylin and eosin (x200 magnification). A. Normal group with healthy heart tissue. B. Control group with necrosis of cardiomyocytes and inflammatory cell infiltration. C. AM 71 mg/kg treated group with necrosis of cardiomyocytes. D. AM 142 mg/kg treated group with fewer inflammatory cells in the myocardial tissue. E. AM 284 mg/kg treated group with inflammatory cells in the myocardial tissue.

Discussion

MI accounts for a significant proportion of the deaths from the CVD. MI is a highly prevalent ischemic condition characterized by tissue necrosis that develops due to an imbalance between oxygen needs and actual supply [18]. This imbalance leads to cardiac ischemia and the degeneration of cardiomyocytes [3]. The present study was to determine the protective effect of AM against MI induced by ISO in rats. The model of myocardial infarction induced by the ISO was performed according to the methodology of Priya Saxena in (2009) [18].

ISO-induced MI is extensively employed in in vivo animal models for the experimental evaluation of cardioprotective agents as it is clinically pertinent in recapitulating the features of human MI [19]. Subcutaneous administration of ISO causes an imbalance between oxygen supply and demand of the cardiomyocytes through increasing the chronotropism and inotropism essential to myocardial function and results in the overload of the myocardium [19, 20].

When the cell membrane ruptures, cytosolic enzymes, including CK, LDH, AST, and ALT, leak from the damaged tissue into the bloodstream. The amount of these cellular enzymes in the serum reflects alterations in the cardiac myocytes' plasma membrane integrity and permeability [21]. In our study, rats receiving ISO showed significant increases in the CK, AST, and LDH levels in serum. These indications of ISO-induced necrotic damage of the myocardium and leakiness of the plasma membrane's results were consistent with previous reports [21-23]. However, pretreatment with AM resulted in decreased serum levels of CK, AST, and LDH, demonstrating that AM contributed to the membrane integrity, thereby decreasing these enzymes' leakage. Polyphenolic and flavonoids are important constitutive antioxidants found in the AM [24] and confer protective effects on the liver by preserving the membrane integrity [25]. We observed that these antioxidants can reduce oxidative cardiac injury, as well as inhibiting the leakage of these enzymes from the myocardium.

The metabolites and auto-oxidation resulting from ISO damage generate free radicals involved in the pathogenesis of myocardial ischemia [26]. After the administration of ISO, a strong decline in the heart's endogenous antioxidant system's activities leading to the loss of pro-oxidant/antioxidant balance and oxidative damage [27].

ISO renders the myocardium more vulnerable to lipid peroxidation due to the oxidative degeneration of fatty acids in the myocardial membrane and damages the antioxidant protective mechanisms. Increased formation of MDA is an indication of the severity of the myocardial cellular injury induced by ISO, and this can be linked to altered membrane structure and enzyme inactivation [25]. The decreased MDA levels with simultaneously increased SOD levels in rats pretreated with AM in our study can be reasonably hypothesized to augment the actions of the myocardium's native antioxidant defense mechanism. According to Zhou, in 2000, the protective effects of the different components isolated from Astragalus membranaceus Bunge on cardiac function during ischemia-reperfusion may be related to improving energy metabolism, scavenging the oxygen free radicals, and inhibiting the production of free radicals in the ischemic myocardium [14].

The histopathological examination of the myocardial tissue of normal control rats clearly illustrated the integrity of the myocardial cell membrane and no inflammatory cell infiltration was observed. The ISO-induced control group rat's myocardium showed necrosis of the cardiomyocytes, cell nucleus disappearance, macrophage accumulation, and connective tissue formation in the injured myocardium, which had few blood vessels. Pretreatment of the AM 142 mg/kg showed a decreased area of necrosis and inflammatory cells in the infarcted myocardial tissue. AM's protective effect might have been mediated through an AM-induced increase in the myocardial antioxidant enzyme activities.

Despite having a small sample, our findings show that the Astragalus mongholicus Bunge extract exhibits cardioprotective effects against isoproterenol-induced myocardial infarction in rats – possibly through augmenting the actions of the myocardium's antioxidant defenses. However, our future study will examine anti-inflammatory and anti-apoptotic activity in myocardial infarction models. The anti-apoptotic activity of Astragalus mongolicus needs to be investigated using immunohistochemical localization of Bax and Bcl-2 proteins and TUNEL staining. These results will provide a basis for studying the cardioprotective mechanisms of Astragalus mongholicus Bunge, which may allow us to develop a new therapeutic approach to prevent and treat ischemic heart disease.

Conclusions

Our results suggest that the extract of Astragalus mongholicus Bunge exhibits cardioprotective effects against isoproterenol-induced myocardial infarction in rats. The AM's protective effect occurs by protecting the myocardium's native superoxide dismutase levels, reducing the levels of malondialdehyde and serum marker enzymes LDH, AST, and CK. Moreover, the extract exhibited antioxidant activity in the AM treated groups.

Conflict of Interest

The authors declare no conflict of interests.

Acknowledgments

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