Protective effect of *Terminalia chebula* against lysosomal enzyme alterations in isoproterenol-induced cardiac damage in rats

Subramaniyan Suchalatha MSc MPhil PhD, Chennam Srinivasulu Shyamala Devi MSc PhD DSc

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BACKGROUND: *Terminalia chebula* is an ayurvedic drug recommended for the treatment of heart diseases. Earlier studies by the authors validated the beneficial cardioprotective effect of *T chebula* against isoproterenol-induced myocardial infarction.
OBJECTIVES: To evaluate the therapeutic efficacy of *T chebula* in protecting against isoproterenol-induced lysosomal membrane damage.
METHODS: Lysosomal enzyme activities from the serum, heart and lysosomal fractions were determined. The triphenyltetrazolium chloride assay was used to confirm the protective effect of *T chebula* on the myocardium.
RESULTS: Isoproterenol administration produced significant cardiac damage (as seen by the triphenyltetrazolium chloride assay) and significantly altered lysosomal enzyme activities. Pretreatment with an ethanol extract of *T chebula* was found to retain near normal activities of lysosomal enzymes in rats given *T chebula* or *T chebula* plus isoproterenol compared with rats given isoproterenol alone.
CONCLUSIONS: Pretreatment with *T chebula* extract stabilizes the lysosomal membrane and, thus, may have prevented myocardial damage.

Key Words: *Isoproterenol; Lysosomes; Myocardial injury; Rats;* T chebula

Isoproterenol is a synthetic agonist and catecholamine long known to cause severe stress in the myocardium that results in infarct-like myocardial necrosis in rats (1). Isoproterenol-induced necrosis is a multifactorial condition involving relative hypoxia, an effect on coronary microcirculation, membrane permeability and the excessive formation of free radicals. Oxygen free radicals generated during ischemia damage the myocardium through the release of lysosomal enzymes (2). Therefore, isoproterenol-induced myocardial infarction serves as a well-standardized model to study the anti-ischemic effects of *Terminalia chebula* (Retz), a plant native to India.

According to an ancient treatise on ayurvedha (3), *T chebula* figures prominently among the list of indigineous remedies advocated for the treatment of cardiac diseases. Studies have demonstrated that *T chebula* exhibits a wide range of biological activities, including cardioprotective (4), 'antivata' or antispasmodic (5), antioxidant (6), free radical scavenging (7) and hypolipidemic properties (8).

Compounds that scavenge for free radicals and have membrane stabilizing potential are reported to be effective in ameliorating the progress of biochemical tissue injury (9). Therefore, the present study sought to evaluate whether pretreatment with *T chebula* extract exerts a protective effect against isoproterenol-induced alterations in lysosomal membrane stability and myocardial tissue damage.

METHODS

Chemicals and reagents

Isoproterenol, ethanol, bovine serum albumin, *p*-nitrophenyl-N-acetyl-beta-D-glucosaminide, *p*-nitrophenyl-beta-D-glucuronide, *p*-nitrophenyl-beta-D-glucosaminidase, triphenyltetrazolium chloride (TTC) and *p*-nitrophenol were obtained from Sigma-Aldrich Company (USA). All other chemicals used were of the highest purity.

T chebula

Powder from the fruit of *T chebula* was a gift from Rohini Herbal Research Institute Private Limited (Chennai, India). *T chebula* powder (1 kg) was soaked in 95% ethanol for seven days with intermittent shaking. The solvent was then filtered with Whatman 1 filter paper (Whatman, India). The filtrate was evaporated under a vacuum drier, and the resultant brown residue was stored at —4°C until further use. Weighed amounts of residue were dissolved in 0.9% saline for experimental use.

Animals

Adult male albino rats (Wistar strain) weighing 120 g to 150 g were obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai. The rats were fed with commercial pellet rat chow (Hindustan Lever Limited, India) and given water ad libitum. They were maintained under standard laboratory conditions, with a 12 h light and dark cycle. The study was conducted according to the guidelines of the human/animal ethics committee (University of Madras, India).

Experimental protocol

Preliminary studies were performed to find the dose of *T chebula* that would be most effective against isoproterenol-induced cardiac damage based on the activities of lactate dehydrogenase (LDH) and creatine kinase. Different doses of *T chebula* extract, ranging from 250 mg/kg body weight to 1 g/kg body weight were administered at time intervals of 15, 21 and 30 days. The optimal cardioprotective effect of *T chebula* was observed at a dose of

Department of Biochemistry, University of Madras, Guindy Campus, Chennai, India
Correspondence: Dr Chennam Srinivasulu Shyamala Devi, Old Number 62, New Number 66 II Main Road, Gandhi Nagar, Adyar, Chennai-20, India. Telephone 04-42-441-2575, fax 91-44-235-2494, e-mail cssdevi@yahoo.com

TABLE 1
Effect of *Terminalia chebula* extract on the activities of lysosomal enzymes in the sera of control and experimental rats

	Group I (control)	Group II (isoproterenol injected)	Group III (*T chebula*)	Group IV (*T chebula* plus isoproterenol)	ANOVA F value
Beta-D-glucuronidase*	8.90±0.70	14.00±1.07§	8.50±0.44¶	10.03±0.91**	50.21
Beta-D-glucosidase*	9.01±0.33	15.01±0.97§	9.01±0.81¶	10.80±1.50**	48.20
Beta-N-acetyl-glucosaminidase*	18.00±1.30	27.30±2.12§	17.50±0.75¶	25.80±2.19††	53.70
Cathepsin D†	14.00±1.21	20.00±2.40§	13.91±1.24¶	16.08±1.29**	18.34
Acid phosphatase‡	80.23±8.80	112.25±8.86§	78.26±7.65¶	89.11±7.90**	21.02

*Enzyme activity expressed in: *p-nitrophenol liberated (µmol/h/100 mg protein); †Tyrosine liberated (µmol/h/100 mg protein); and ‡p-nitrophenol liberated (µmol/min/100 mg protein). §Significantly different compared with group I (P<0.01); ¶Not statistically different compared with group I. **Significantly different compared with group II (P<0.01). ††Significantly different compared with group II (P<0.05) (Bonferroni's Multiple Comparison Test). Values are expressed as mean ± SD for six rats in each group*

TABLE 2
Effect of *Terminalia chebula* extract on the activities of lysosomal enzymes in the heart homogenate of control and experimental rats

	Group I (control)	Group II (isoproterenol injected)	Group III (*T chebula*)	Group IV (*T chebula* plus isoproterenol)	ANOVA F value
Beta-D-glucuronidase*	22.43±2.94	34.88±5.29§	23.23±3.29¶	25.36±3.18**	13.69
Beta-D-glucosidase*	15.20±1.01	28.20±1.41§	15.12±1.03¶	19.39±0.58**	204.74
Beta-N-acetyl-glucosaminidase*	47.64±4.42	67.63±7.89§	43.30±4.17¶	50.27±7.52**	17.02
Cathepsin D†	28.75±3.88	46.34±3.75§	27.22±3.39¶	33.56±2.92**	36.68
Acid phosphatase‡	120.03±11.82	155.48±14.36§	113.52±12.00¶	135.48±14.36**	4.53

*Enzyme activity expressed in: *p-nitrophenol liberated (µmol/h/100 mg protein); †Tyrosine liberated (µmol/h/100 mg protein); and ‡p-nitrophenol liberated (µmol/min/100 mg protein). §Significantly different compared with group I (P<0.01); ¶Not statistically different compared with group I. **Significantly different compared with group II (P<0.01) (Bonferroni's Multiple Comparison Test). Values are expressed as mean ± SD for six rats in each group*

500 mg/kg body weight for 30 days (data not shown). This dose was therefore used for further studies. The rats were divided into four groups of six rats: 'normal' rats (group I); rats administered isoproterenol (200 mg/kg body weight, subcutaneous, given twice with 24 h in-between) (group II); rats pretreated with *T chebula* extract (500 mg/kg body weight, orally, given daily for 30 days) (group III); and rats pretreated with *T chebula* extract (500 mg/kg body weight, orally, given daily for 30 days) and administered isoproterenol (200 mg/kg body weight, subcutaneous, given twice with 24 h in-between) at the end of the pretreatment period (group IV).

At the end of the experimental period, the rats were anesthetized with pentobarbital sodium (35 mg/kg body weight, intraperitoneally). Blood was drawn from the external jugular vein and the serum was separated using a Biofuge Stratos centrifuge at 2500 *g* (Heraeus/Kendro, Germany). The rats were sacrificed 60±5 s after the injection. The hearts were excised, washed in an ice cold 0.9% saline solution, blotted with filter paper and weighed. A section of the heart tissue was used to determine the activities of lysosomal enzymes.

Lysosomal fractions were isolated using the method of Wattiaux (10). The activities of the lysosomal enzymes were determined for beta-D-glucuronidase using the method of Hultberg et al (11); beta-D-glucosidase using the method of Conchie et al (12); beta-N-acetyl-glucosaminidase using the method of Moore and Moris (13); cathepsin D using the method of Sapolsky et al (14); and acid phosphatase using the method of King (15). The lysosome pellet was suspended in 1.15% KCl and used for the estimation of enzyme activity.

TTC assay

A section of the heart tissue was used for the TTC assay as described by Lie et al (16). The myocardium of the rat was frozen immediately after removal. The ventricle portion of the heart was excised, weighed, sliced into 1 mm segments and incubated in a 1% TTC solution at 37°C for 20 min. The weight of the infarcted tissue was expressed as a percentage of the total ventricular weight.

Statistical analysis

The data were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. The results from the experimental groups were compared with their respective control group. $P<0.05$ was considered statistically significant. The infarct size was analysed using one-way ANOVA followed by Student's *t* test, and $P<0.001$ was considered statistically significant.

RESULTS

The activities of lysosomal hydrolases in the sera of control and experimental groups are shown in Table 1. Significant elevations in the activities of beta-D-glucuronidase, beta-D-glucosidase, beta-N-acetyl-glucosaminidase, cathepsin D and acid phosphatase were observed in isoproterenol-administered rats (group II) compared with the control rats (group I). In isoproterenol-administered rats pretreated with *T chebula* extract (group IV), significantly lower activities in serum lysosomal hydrolases were observed compared with rats injected with isoproterenol alone (group II) ($P<0.01$).

The activities of the lysosomal hydrolases from heart tissue homogenates of the control and experimental groups are shown in Table 2. Significant increases in the activities of beta-D-glucuronidase, beta-D-glucosidase, beta-N-acetyl-glucosaminidase, cathepsin D and acid phosphatase were observed in the heart tissue of isoproterenol-administered rats compared with the control rats (group I) ($P<0.01$). *T chebula* extract pretreatment (group IV), however, resulted in significantly lower activities of heart lysosomal enzymes compared with rats given isoproterenol alone (group II).

TABLE 3
Effect of *Terminalia chebula* extract on the activities of lysosomal enzymes in the lysosomal fraction of control and experimental rats

	Group I (control)	Group II (isoproterenol injected)	Group III (*T chebula*)	Group IV (*T chebula* plus isoproterenol)	ANOVA F value
Beta-D-glucuronidase*	41.94±5.10	22.88±2.64§	42.58±4.66**	37.58±3.48††	30.23
Beta-N-acetyl-glucosaminidase*	52.38±5.47	31.66±4.00§	49.63±4.47**	45.55±4.45††	23.66
Cathepsin D†	70.55±7.56	58.38 ±5.44¶	69.76±6.77**	67.26±7.43‡‡	5.03
Acid phosphatase‡	121.36±10.82	83.39±5.38§	122.53±11.04**	105.03±11.75††	19.51

*Enzyme activity expressed in: *p-nitrophenol liberated (µmol/h/100 mg protein); †Tyrosine liberated (µmol/h/100 mg protein); and ‡p-nitrophenol liberated (µmol/min/100 mg protein). §Significantly different compared with group I (P<0.01). ¶Significantly different compared with group I (P<0.05). **Not statistically different compared with group I. ††Significantly different compared with group II (P<0.01). ‡‡Significantly different compared with* group II *(P<0.05) (Bonferroni's Multiple Comparison Test). Values are expressed as mean ± SD for six rats in each group*

The activities of lysosomal hydrolases in the lysosomal fractions of the control and experimental groups are shown in Table 3. The activities of lysosomal hydrolases were found to be significantly decreased in the lysosomal fraction of group II rats compared with group I rats. The activities of these enzymes were maintained at near normal levels in rats pretreated with *T chebula* extract (group IV). Rats pretreated with *T chebula* extract alone (group III) showed no significant change in lysosomal hydrolases activities in the serum, heart and lysosomal fractions compared with controls.

TTC assay

TTC macroscopic enzyme-mapping assay of sections of heart from control and experimental rats (Figure 1) is direct evidence of myocardial necrosis. Figure 1A shows a section of heart from a control rat with viable myocardial tissue stained to indicate the presence of LDH and intact myocardial tissue. Figure 1B shows a section of heart from an isoproterenol-administered rat. Necrotic tissues are clearly visible as light gray patches. One of the characteristic features of isoproterenol administration is the loss of LDH activity from myocardium, and may reflect the consequence of cellular injury. Figure 1C , a section of heart tissue from a rat pretreated with *T chebula* extract alone, shows results similar to that of the control. Figure 1D shows a section of heart tissue from a *T chebula*-pretreated rat administered isoproterenol. A major portion of the heart tissue stained positive for viability, indicating that the prior oral administration of *T chebula* extract may have prevented membrane damage by isoproterenol, thereby retaining near normal myocardial membrane structural and functional integrity. The effect of *T cheblua* on myocardial infarct size of control and experimental rats is shown in Table 4.

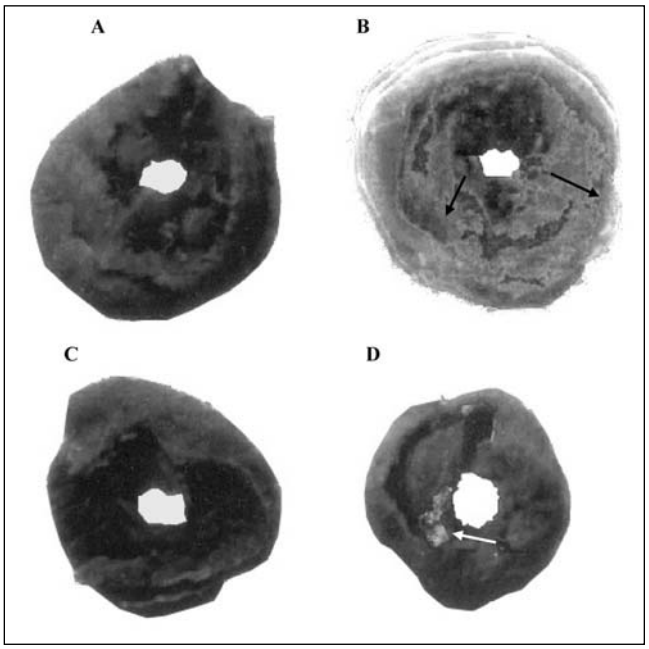

Figure 1) *Representative results from the triphenyltetrazolium chloride assay of sections of heart from the control (n=6) and experimental rat (n=6) groups:* (**A**) *Control rats (group I);* (**B**) *Isoproterenol-administered rats (group II);* (**C**) Terminalia chebula*-pretreated rats (group III);* (**D**) T chebula*-pretreated plus isoproterenol-administered rats (group IV). Arrows indicate patches of necrotic tissue*

TABLE 4
Effect of *Terminalia chebula* extract on the myocardial infarct size of control and experimental rats

	Necrotic tissue (% of ventricle)	ANOVA F value
Group I (control)	0.00±0	
Group II (isoproterenol injected)	28.1±2.8*	131.3
Group III (*T chebula*)	0.00±0	
Group IV (*T chebula* plus isoproterenol)	9.1±0.3	

**Statistically significant from group I (P<0.001; one-way ANOVA followed by Student's* t *test). Values are expressed as mean ± SD for six rats in each group*

DISCUSSION

Lysosomal enzymes are important mediators of acute and chronic inflammatory diseases, and can cause damage to connective tissue (17). Alterations in the activity of lysosomal enzymes have been observed in patients with myocardial infarction (18) and in experimental animal models (19). Therefore, considerable attention has been focused on lysosomal enzyme alterations that may accompany ischemic or hypoxic myocellular damage (20,21).

Macickova et al (22) observed that isoproterenol is able to induce changes in lysosomal enzyme activity both in vivo and in vitro. Isoproterenol-induced myocardial infarction results in increased activities of the lysosomal hydrolases in the serum and heart tissue, and a decrease in their activities in the lysosomal fraction. This may be responsible for myocardial cellular injury and death in the ischemic state of the heart (22,23). The findings in the present study support this.

It has been previously reported that ischemia produces rapid accumulation of lactic acid and other metabolic acids (which

lowers intracellular pH), and decreases in ATP and the active accumulation of free fatty acids (25,26). In turn, these events produce a reduction in membrane integrity, which initiates the release of lysosomal enzymes. The leakage of the enzymes from the enclosed sacs leads to intracellular dysfunction, disruption of potential substrates (27) and organelles (mitochondria [28], sarcolemma, etc [29,30]), and autolysis of myocardial cells (31). The significantly lower activities of the enzymes in the lysosomal fraction in isoproterenol-administered rats correlates well with these findings. *T chebula* extract pretreatment led to the retainment of near normal activity of the enzymes in the lysosomal fraction, suggesting the stabilization of the lysosomal membrane by *T chebula* extract.

The increased activities of cathepsin D and glycohydrolases in heart tissue indicate the possible infiltration of inflammatory cells at the site of infarction. During myocardial infarction, when myocardial cell death and degeneration occurs, proteolysis of necrotic myocardium occurs with a concomitant influx of inflammatory cells at the infarct margins (32).

In experimental myocardial infarction, decreased lysosomal stability leads to elevated levels of lysosomal enzymes in the extracellular fluid (33), and alters the metabolism of different connective tissue constituents, namely, glycosaminoglycans and glycoproteins (34). The release of lysosomal enzymes contributes to tissue destruction and may be one of the causes of increased focal lesions observed in heart tissue (35). In the present study, the observed increases in the activities of lysosomal enzymes in the serum and heart tissue is an indication of isoproterenol-mediated lysosomal membrane damage.

The release of lysosomal enzymes into the cytoplasm stimulates inflammatory mediators (eg, oxygen radicals and prostaglandin), which then stimulate tissue disruption. Considering this and the well-known lytic action of lysosomal enzymes, it suggests that the damage to the lysosomal membrane and alterations in the fragility of lysosomes may be among the earliest structural alterations that occur during the development of ischemic myocardial injury (22).

Pretreatment with orally administered *T chebula* extract led to the retention of near normal activities of the lysosomal enzymes in the serum and heart tissue. Pretreatment with *T chebula* extract was associated with a decreased release of enzymes from the lysosomal fractions, which could be due to the membrane stabilizing effect of *T chebula* on the lysosomal membrane. *T chebula* has been reported to possess higher anti-inflammatory activity compared with prednisolone (36). *T chebula* has been reported to possess flavonoids which exhibit anti-inflammatory, vasodilatory, lipid peroxidation, antioxidant and free radical scavenging properties (37-39). The antioxidant property is due to *T chebula* extract scavenging for oxygen free radicals, resulting in the preservation of cellular viability serving, secondarily, to preserve lysosomes and, thereby, retaining near normal functioning of the lysosomes.

CONCLUSIONS

Pretreatment with *T chebula* extract may partly impart its cardioprotective effect through lysosomal membrane stabilization, thus preventing myocardial necrosis.

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