

Tribulus terrestris protects rat myocardium against isoproterenol-induced ischemic injury: role of HSP 70 and cardiac endogenous antioxidants

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Abstract

The present study was undertaken to evaluate the cardioprotective activity of *Tribulus terrestris* (Tt), a medicinal herb following isoproterenol (ISP)-induced myocardial injury. The contribution of heat shock protein (HSP) 70, key anti-stress protein, endogenous antioxidants and oxidant-antioxidant balance in attenuating myocardial injury was further studied. Hydroalcoholic extract of Tt {1, 2.5, 5 & 10 mg/kg} were orally fed once a daily to Wistar rats for 21 days. On the 20th and 21st day, both control (ISP control) and Tt fed rats were challenged with ISP (85 mg/kg, s. c. two doses at 24h intervals) induced myocardial necrosis. Histopathological evaluation, cardiac marker enzyme: Creatinine phosphokinase (CPK) and antioxidative parameters: Glutathione (GSH), Thiobarbituric acid reactive substances (TBARS), Catalase (CAT), Glutathione peroxidase (GSHPx) and Superoxide dismutase (SOD) levels were estimated. Tt (2.5 mg/kg) intake *per se* upregulated HSP 70; increased basal SOD, CAT activity ($P < 0.05$) and caused a marked fall in basal TBARS levels ($P < 0.05$) in comparison to sham. Following ISP challenge, significant oxidative stress with evidence of myocardial necrosis was observed in the ISP control group. ISP-induced changes in myocardial SOD, GSHPx and GSH were prevented by both the 2.5 and 10 mg/kg doses of Tt, though cellular injury was minimal with 2.5 mg/kg dose. The results emphasize that pre-treatment with Tt offered significant protection against ISP-induced myocardial necrosis through a unique property of enhancement of endogenous antioxidants, stabilization of cytoskeleton structure which in turn is attributed to HSP 70 expression along with fortified antioxidant defense system.

Introduction

Oxidative stress is the major etiopathologi-

cal factor in ISP-induced myocardial necrosis. A relatively low amount of endogenous antioxidant makes the heart vulnerable to oxidative stress-induced damage.¹⁻³ Considerable efforts have been made in the exploration of the potential for exogenous antioxidants and free radical scavengers to supplement endogenous antioxidant system and limit free radical injury, with mixed success and failure.^{4,5} One reason why exogenous antioxidants have limited success in the prevention of myocardial injury may be due to the inaccessibility of large molecules to the key intracellular sites of oxidative damage. Under such circumstances, other options need to be explored which will help in circumventing this problem, such as, whether, by any means, it is possible to stimulate or augment the endogenous antioxidant defense system of the heart.⁵

A concept is now emerging of 'adaptogenic drugs' drugs that increase non-specific resistance of the users to a variety of stresses. One of the proposed mechanisms of action of such drugs is by enhancing basal cellular endogenous antioxidant enzymes (SOD, CAT, GSHPx), and nucleic acid biosynthesis.^{6,7} Role of heat shock proteins (HSPs), most notably the inducible 70-kDa HSP family member HSP 70, in myocardial adaptation has been well documented. HSP 70 is believed to participate in the protection of the myocardium during cardiovascular stress as well as apoptosis. Up regulation of HSP70 has been associated with the reduction in myocardial apoptosis as it prevents the degradation of nucleolin, an anti-apoptotic protein.⁸ In addition, studies demonstrated that HSP 70 enhanced the mitochondrial energetic capacity and increased the tolerance to myocardial injury.^{9,10} This unique property of *augmenting endogenous antioxidants and HSP 70* has been explored in the present study.⁵

Tribulus terrestris (Tt) has long been a constituent in tonics in Indian ayurveda practice, where it is known by its Sanskrit name, *gokshura*. It is also used as an aphrodisiac, diuretic and nervine in Ayurveda, and in Unani, another medical system of India. The saponins (furostanol) and flavanoids present in the leaves and fruits are the active principles responsible for its vasodilatory and diuretic properties.^{10,11} Since the first report of cardiac actions of Tt was published in 1976,¹² there has been a paucity of research investigating the potential benefits of using Tt as a cardioprotective agent. Recently, Guo et al., evaluated the effects of Tt saponins on ventricular remodeling after myocardial infarction in hyperlipidemic rats.¹³ However, the cardioprotective and adaptogenic potential of Tt in the setting of ISP induced myocardial necrosis has not been studied before. By virtue of its adaptogenic and antioxidant properties, Tt may offer protection against oxidative stress-

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induced by ISP, a catecholamine known to have deleterious effects on the myocardium.⁵ With this point of view the study was designed to provide scientific data and validate the cardioprotective effects of Tt as well as to delineate its mechanism of action.

Immunohistochemical localization of HSP 70, indices of oxidant-antioxidant balance: Thiobarbituric acid reactive substances (TBARS), endogenous antioxidant: GSH and antioxidant enzymes (SOD, CAT, GSHPx) were incorporated into the study design. Cardioprotection was confirmed by assessing the severity of pathological changes and myocardial CPK.

Materials and methods

Experimental animals

Adult male Wistar rats, 10 to 12 weeks old, weighing 150 to 200 g were used in the study. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee and conforms to the Indian National Science

Academy Guidelines for the Use and Care of Experimental Animals in Research. Animals were obtained from the Animal Facility of Mahatma Gandhi Mission Medical College, Navi Mumbai, India. The rats were housed in an air-conditioned room and were kept in standard laboratory conditions under natural light and dark cycles (approximately 14 h light/10 h dark) and maintained at humidity 60±5% and an ambient temperature of 25±2°C. The animals were allowed free excess to standard diet and tap water *ad libitum* and allowed to acclimatize for one week before the experiments.

Chemicals

All Chemicals were of analytical grade, purchased from Sigma Chemical Co., St Louis, USA. The ABC staining kit and secondary antibodies (Anti mouse IgG) were procured from Santa Cruz Biotechnology, USA. HSP 70 mouse monoclonal IgG1 primary antibody was procured from Biogenex Life Sciences Private limited, India.

Hydro-alcoholic lyophilized extracts of *Tribulus terrestris* was procured from Dabur Research Foundation, India. The multiple solvent (methanol: isopropyl alcohol: acetone) extraction procedure was used to prepare the extract by the supplier. The whole plant was used for extraction. The extractive values (taking 1 gm sample) in water was 64.15% w/w and in methanol was 53.94 w/w. pH of 1% w/v aqueous solution was 8.23 and loss on drying value at 105°C by infrared balance was 3.1 w/w. The total ash content was 14.06% w/w. Total saponin content (on dried basis) was not less than 20-45% w/w by HPLC.

Experimental groups and treatment protocol

The animals were assigned to the following experimental groups. There were ten animals in each group.

Baseline evaluation protocol

In this group healthy experimental animals were used to evaluate baseline values of various parameters investigated in this study i.e rats without any pathologic challenge to the heart

Group 1 – Saline control group (Sham)

Rats were administered 0.9% normal saline per orally using a feeding cannula for 21 days and then sacrificed on the 22nd day.

Group 2 – *Tribulus terrestris* control group

This group was divided in four subgroups. Hydro-alcoholic extract of Tt was dissolved w/v in 0.9% normal saline administered orally to healthy experimental rats once daily for 21 days at the doses 1 mg/kg (Tt-1), 2.5 mg/kg (Tt-2.5), 5 mg/kg (Tt-5) and 10 mg/kg (Tt-10).

Isoproterenol (ISP)-induced myocardial necrosis protocol

Group 3 – Isoproterenol group - ISP control

The rats were administered 0.9% normal saline for 21 days and in addition administered ISP (85 mg/kg, subcutaneously) on 20th and 21st day at an interval of 24 h.

Group 4 - *Tribulus terrestris* treated group

It was administered orally to healthy experimental rats once daily for 21 days and thereafter, the rats, were challenged with ISP (85 mg/kg) on 20th and 21st day at an interval of 24 h. This group was further divided into three subgroups:

Group 4a -ISP + 1 mg/kg (ITt-1)

Group 4b - ISP + 2.5 mg/kg (ITt-2.5)

Group 4c - ISP + 5 mg/kg (ITt-5)

Group 4d - ISP + 10mg/kg (ITt-10)

Experimental parameters studied

Biochemical studies

A ten-percent homogenate of myocardial tissue was prepared in 50 mM phosphate buffer, pH 7.4 and an aliquot was used for the assay of TBARS according to the method described by Ohkawa *et al.*,¹⁴ The homogenate was centrifuged at 7000 rpm for 15 minutes and the supernatant was used for the estimation of the glutathione,¹⁵ glutathione peroxidase,¹⁶ superoxide dismutase,¹⁷ Catalase,¹⁸ and protein.¹⁹ Creatinine phosphokinase was estimated spectrophotometrically using a kit from Randox Laboratories, USA.²⁰

Immunostaining for the Localization of HSP Proteins

Immunohistochemistry using the mouse anti-cleaved monoclonal heat shock protein IgG1 antibody was performed on deparaffinized tissue sections using a routine avidin–biotin–immunoperoxidase “technique”. Before incubation with the primary rabbit polyclonal antibody (1:1000 dilution), tissue sections were subjected to heat-induced epitome retrieval by incubation in a pH 8.0 0.01 M EDTA solution for 10 min in a vegetable steamer, followed by 20-min cool-down and treatment with 3% hydrogen peroxide before antibody application. Bound antibodies were detected using HRP-Straptavidin complex. The target protein (HSP) was visualized by incubation in peroxidase substrate complex and DAB (3,3' diaminobenzidine) as the chromogen. Counterstaining was performed with Meyer's hematoxylin. All descriptions and the pictures given in the manuscript are based on specific staining as adjusted against the positive and negative controls.

Histopathological studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. Cross sections (5 µm thick) of the fixed myocardial tissues were cut. These sections were stained with hematoxylin and eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the myocardium. The degree of necrosis was graded and scored.

Statistical analysis

All numerical data in text, figures and tables are expressed as the mean + SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) or repeated measures ANOVA, followed by the Bonferroni post hoc test. Differences were considered statistically significant at P<0.05.

Results

Isoproterenol (ISP)-induced myocardial necrosis protocol

Biochemical parameters without ISP-induced myocardial necrosis

Oral feeding of Tt (2.5 and 10 mg/kg) *per se* to healthy experimental rats for 21 days, augmented basal SOD and CAT activity (P<0.05) as compared to sham baseline values (Table 1). Tt at 1, 5 & 10 mg/kg did not alter basal TBARS levels; only 2.5 mg/kg dose was able to significantly reduce baseline lipid peroxidation as evidenced by fall in basal TBARS level (P<0.05) in comparison to sham. In addition, no significant increase in the basal GSH levels and GSHPx activity was seen in any of the Tt control groups in reference to sham group (Table 1).

Biochemical parameters following ISP-induced myocardial necrosis

ISP induced myocardial necrosis resulted in a significant depletion of myocardial GSH (P<0.05), fall in myocardial antioxidant enzymes {SOD (P<0.01), CAT (P<0.01), GSHPx (P<0.05), CPK (P<0.01)} and increase in lipid peroxidation as evidenced by elevated {TBARS levels (P<0.01)} as compared to sham (Table 2). Tt (2.5 and 10 mg/kg) doses significantly inhibited lipid peroxidation (P<0.05, Figure 1) and preserved membrane integrity. In addition, significant restoration of myocardial CPK enzyme with Tt-2.5 mg/kg (P<0.01) and Tt-10 (P<0.05) was observed in reference to ISP control. Subsequent to ISP induced myocardial necrosis, Tt(2.5 and 10 mg/kg) significantly protected SOD (P<0.05) and GSHPx(P<0.05) (Figure 2). However, none of the doses studied restored GSH levels as compared to ISP control (Table 2).

Heat shock protein 70 expression following Tt pre-treatment

HSP70 immunostaining in the myocardium was very faint in sham operated controls (4.2 + 4.2%; Figure 3A). In the Tt (1 mg/kg and 5mg/kg) control group no significant change in the expression of basal HSP 70 (6.2 + 2.1%; 4.8 + 3.1%) respectively was observed as compared to sham group. In both the groups, myocytes were weakly positive for HSP70. However, in the Tt (2.5 mg/kg) control group oral pretreatment for 21 day resulted in significant up-regulation (35.2 + 3.1%) of basal HSP 70 expression (Figure 3B). Similarly, in the Tt (10 mg/kg) control group strong positive staining was seen in microvessels as well as myocytes (25.2+3.1%) as compared to sham group (Figure 3C).

Histopathological studies following ISP-induced myocardial necrosis

Microscopic histology revealed that the sham group was characterized by an organised pattern and shows normal architecture of the myocardium.

However, in the rat sections of the ISP control group, myonecrosis with fibroblastic proliferation, infiltration of inflammatory cells, marked intramyocellular edema, besides vacuolar degeneration and rounded nuclei as compared to sham-operated group

was observed. Tt(2.5 and 10 mg/kg) treatment prevented myonecrosis, infiltration of inflammatory cells, edema and vacuolar changes as compared to the ISP control. However, in the Tt-1 and Tt-5 treated groups of the study protocol the degree of edema and necrosis was nearly comparable to that of ISP control group with similar morphological changes (Table 3).

Discussion

Nature has been a source of medicinal treatments for thousands of years and plant-derived products continue to play an essential role in the primary health care of about 80-85% of the world's population.^{21,22} There has been an upsurge in the use of medicinal plants for the

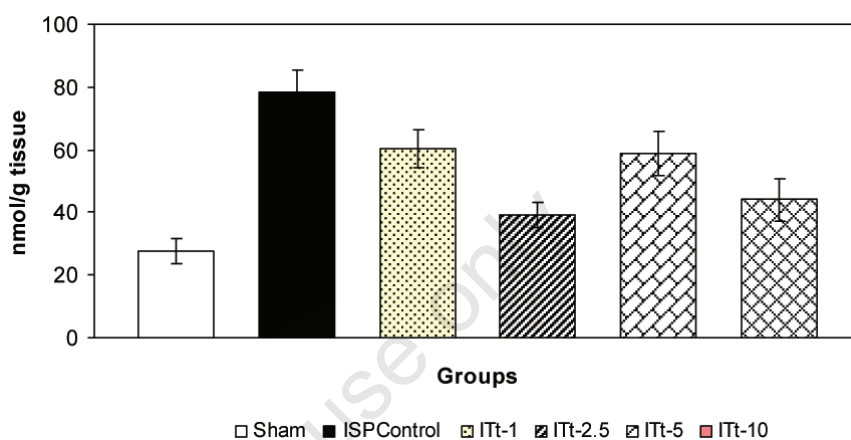


Figure 1. Myocardial TBARS levels in the different experimental groups. ##P<0.01 Vs Sham, *P<0.05 Vs ISP Control.

Table 1. Biochemical parameters in the different groups.

	Sham	Tt-1	Tt-2.5	Tt-5	Tt-10
GSH (umol/g tissue)	4.03±0.3	4.4±0.6	5.4±0.6	4.23±0.4	4.16±0.2
TBARS (nmol/g tissue)	29.73±5.7	27.48±3.4	18.33±1.3*	25.48±3.4	22.8±0.9
GSHPx (IU/mg protein)	0.33±0.1	0.38±0.1	0.38±0.1	0.41±0.1	0.43±0.1
SOD (IU/mg protein)	7.94±2.9	7.12±0.4	28.04±1.2**	7.12±0.4	18.37±1.7**
CAT (IU/mg protein)	21.1±3.1	26.0± 5.6	54.8± 8.0*	26.0± 5.6	46.3±9.6*
CPK (IU/mg protein)	6.7±1.4	7.7±0.8	7.3±0.8	7.1±0.4	7.4±0.5

*P<0.05, **P<0.01 vs sham group. GSH(Glutathione);TBARS(Thiobarbituric acid reactive substances) & Glutathione peroxidase(GSHPx), Superoxide dismutase(SOD), Catalase(CAT) and CPK(Creatinine phosphokinase). One unit of catalase activity represents 1 mol of H₂O₂ decomposed / min. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine. One unit of enzyme activity is defined as 1 nmol of NADPH utilized per min at 37°C. One unit of CPK is defined as the amount of enzyme that will transfer 1µmol of phosphate from phosphocreatine to ADP per min at pH 7.4 at 30°C.

Table 2. Biochemical parameters in the different groups.

Groups/Biochemical Parameters (units)	SOD (IU/mg protein)	CAT (IU/mg protein)	GSHPx (IU/mg protein)	GSH (umol/g tissue)
Sham	7.94±1.93	21.06±3.05	0.33±0.09	4.03±0.3
ISP Control	3.36±0.97##	11.92±1.98##	0.19± 0.08#	2.3±0.1#
ITt-1	4.01±0.42	14.05±6.36	0.22±0.03	2.6±0.3
ITt-2.5	5.51±1.24*	16.05±6.36	0.38±0.05*	3.02±0.1
ITt-5	4.01±0.42	15.43±4.91	0.26±0.03	2.8±0.2
ITt-10	5.75±1.29*	15.61±6.70	0.29±0.10	2.99±0.4

##P<0.01 vs Sham; *P<0.05 vs ISP Control. Glutathione peroxidase (GSHPx), Superoxide dismutase (SOD) and Catalase(CAT). One unit of catalase activity represents 1 µmol of H₂O₂ decomposed / min. One unit of SOD is defined as the amount of enzyme required to produce 50 % inhibition of epinephrine. One unit of enzyme activity is defined as 1 nmol of NADPH utilized per min at 37°C.

treatment of various diseases. A concept is now emerging of *adaptogenic drugs* drugs that increase non-specific resistance of the users to a variety of stresses. Adaptogenic' property of various herbs like *Ocimum sanctum*, *Bacopa monniera* and *Withania somnifera*, first time reported by Brekhman and associates in *Eleuthrococcus* and *Panax ginseng* has already been reported in various experimental studies.^{8,23} These herbs allow one to adapt to a variety of heightened stressful circumstances. Although the exact mechanism of such adaptation is presently not known, it has been proposed that these drugs may act by inducing a number of antioxidant enzymes such as SOD, CAT, GSHPx and antioxidants such as GSH, proteins HSP in the heart.^{7,24} The study was designed to evaluate the a) adaptogenic effects of Tt (based on modulation of myocardial antioxidant system and HSP 70 expression) and b) cardioprotective potential of the medicinal herb following ISP induced myocardial necrosis.

The present study for the first time demonstrates the adaptogenic property of Tt. Oral administration of Tt per se to healthy experimental animals resulted in a significant increase in CAT, SOD activity with 2.5 and 10 mg/kg doses and inhibition of basal lipid peroxidation with Tt (2.5 mg/kg) doses. However, none of the doses of Tt evaluated in this study resulted in significant augmentation of myocardial GSH content and GSHPx activity. Any increase in SOD activity is beneficial in the event of increased free radical generation.²³ However, it has been reported that an augmented SOD activity, without a concomitant rise in the activity of CAT and/or GSHPx might be detrimental, since SOD activity, generates hydrogen peroxide as a metabolite, which is more cytotoxic than oxygen radicals and must be scavenged by CAT or GSHPx. A simultaneous increase in CAT and/or GSHPx activity is essential for an overall beneficial effect of an increased SOD activity.²⁵ Thus, simultaneous increase in myocardial SOD and CAT activities observed in the present study with administration of Tt may contribute to the adaptogenic effects of Tt pretreatment.

HSPs constitute an endogenous stress response for myocardial adaptation.²⁶ There is mounting evidence that cardiac cells synthesize HSPs in response to a variety of stresses and that this stress response may result in protection from subsequent ischemic exposure. For example, several investigators have demonstrated increased cardiac HSP synthesis in response to hyperthermia, hemodynamic overload, cardiac transplantation hypoxia, and ischemia.^{8,9,27} These cytoprotective effects of HSPs make them tempting targets for therapeutic interventions and myocardial adaptation. HSP induction can be achieved by a multitude of different stimuli, among them hyper-

thermia, hypoxia, ischemia, oxidative stress, and various drugs.⁸

In the present study, a nine-six fold increase in the basal expression of HSP 70 was observed in the Tt (2.5 and 10mg/kg) control groups. HSP 70 in the heart was exclusively expressed in myocytes and endothelial cells. Within these cells HSP 70 was co-localized in the nucleus and in the cytosol. These observations are analogous with those of other investigators concerning the apparent role of HSPs in protecting myocardial cells from stress as well as apoptosis.²⁸ Upregulation of HSP70 is associated with the reduction in myocardial apoptosis as it prevents the degradation of

nucleolin, an anti-apoptotic protein.^{9,29} In addition, HSP 70-provides enhancement of mitochondrial energetic capacity and increased tolerance to myocardial injury. Protection by HSP 70 against myocardial dysfunction may be partially due to enhancement of mitochondrial energetics.⁸

Induction of HSP 70 and antioxidant enzymes in the Tt (2.5 and 10 mg/kg) groups also translated into significant myocardial salvaging effects following ISP induced myocardial necrosis. Tt (2.5& 10 mg/kg) treatment significantly restored CPK activity as compared to ISP control group demonstrating the biochemical basis of protective action of Tt. In

Table 3. Light microscopic changes in the different experimental groups.

Groups	Necrosis	Edema	Inflammation
Sham	-	-	-
ISP Control	++++	+++	+++
ITt-1	++++	+++	+++
ITt-2.5	++	++	+
ITt-5	+++	++	+++
ITt-10	++	++	++

The degree of necrosis was graded and scored as follows: Score (-): Absence of any inflammation, edema and necrosis; Score (+): Focal areas of inflammation, edema and necrosis; Score (++) : Patchy areas of inflammation, edema and necrosis; Score (+++) : Confluent areas of inflammation, edema and necrosis; Score (++++): Massive areas of inflammation, edema and necrosis

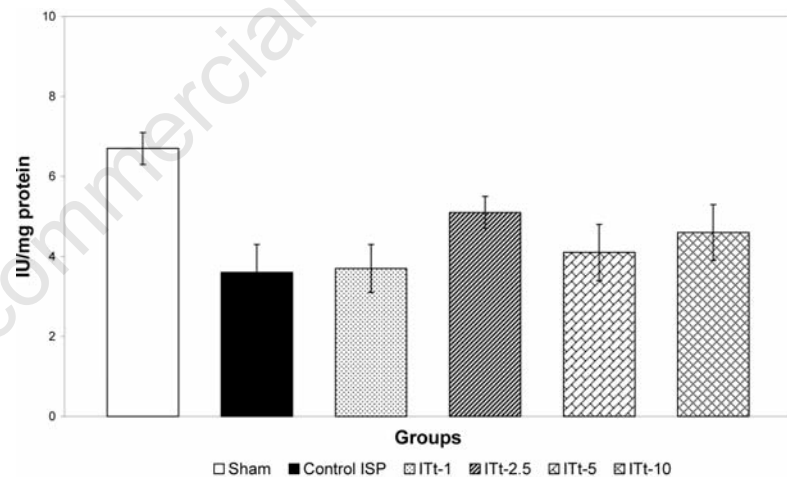


Figure 2. Myocardial CPK activity in the different experimental groups. ##P<0.01Vs Sham, *P<0.05 Vs ISP Control.

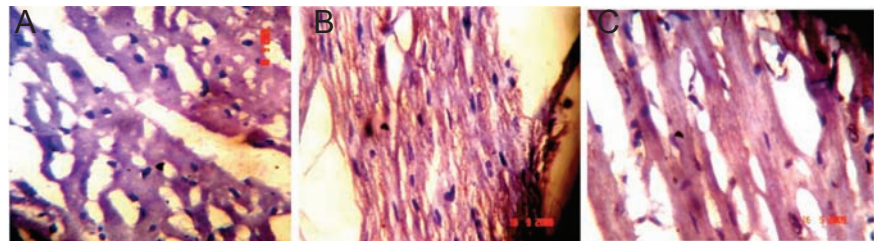


Figure 3. Immunohistochemical findings of HSP70 proteins. A) Non-ischemic myocardium of sham group was weakly positive for HSP 70. B) Upregulation in the expression of HSP 70 as indicated by dark brown positive immunoreactivity is evident in the Tt(2.5 mg/kg) control group as compared to sham group C) In the Tt(10 mg/kg) control groups, significant up regulation of HSP 70 expression as compared to sham group. Figures are representative of 6 separate experiments.

addition, subsequent to ISP induced myocardial necrosis, Tt (2.5 and 10 mg/kg) treatment decreased lipid peroxidation (reduced formation of TBARS from fatty acids). Previously, it has been reported that oral administration of methanolic fraction of Tt fruit extract at dose 6 mg/kg body weight provided protection against the mercuric chloride induced lipid peroxidation in the mice.³⁰ Saponins, the active constituent of Tt inhibit the metabolism of arachidonic acid via the cyclo-oxygenase and lipoxygenase pathways that generates reactive oxygen species; resulting in a decrease in the levels of lipid peroxides.^{13,31} Furthermore, protection against ISP induced oxidative stress in Tt treated rat hearts was evidenced by preservation of endogenous antioxidants enzyme SOD and GSHPx. However, Tt failed to significantly prevent the loss of GSH and CAT enzyme subsequent to ISP challenge. It appears that the major burden of neutralizing the ISP induced oxidative stress was borne by GSH and not by the antioxidant enzymes SOD and GSHPx.

Tt(2.5 & 10 mg/kg) doses significantly prevented myonecrosis subsequent to ISP induced myocardial necrosis via up regulating the expression of basal HSP 70, enhancing the endogenous antioxidant network of the myocardium and maintaining the antioxidant status. This adaptogenic property may contribute to its cardioprotective effect and strengthen the antioxidant defense mechanisms of the heart. Among the various doses evaluated in the present study, Tt at 2.5 mg/kg exhibited optimum cardioprotective activity.

Conclusions

The present study, for the first time demonstrates that Tt (2.5 mg/kg) offered significant protection against ISP induced myocardial necrosis through a unique property of enhancement of basal endogenous antioxidants, heat shock protein and antioxidant property without producing any cytotoxic effects.

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