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Terminalia arjuna (Roxb.) Reverses the Molecular Signature of Fibrosis Induced by Isoproterenol in Rat Heart

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Abstract

Cardiac fibrosis is one of the leading causes of heart failure and therapeutic options are limited. Among various Indian herbal preparations for treating cardiac ailments, the commonly used one is the bark extract of the *Terminalia arjuna* (Roxb) tree but the mechanisms of actions are unknown. To evaluate its therapeutic potential against cardiac fibrosis, male Wistar rats were injected with isoproterenol (5 mg/kg) for 8-12 weeks and the aqueous TA extract was administered orally. Two-dimensional M-mode echocardiography showed partial restoration of cardiac function by TA. Isoproterenol increased the transcript level of pro-fibrotic genes like Col1α, Col3α, Tgf-β1, and CTGF and TA extract significantly attenuated it. TA extract also increased the activities of antioxidant enzymes catalase and SOD; the levels of GSH content as well as reduced the levels of lipid peroxidation. Increased DNA binding activities of redox sensitive transcription factors NF-κB, and AP-1 in isoproterenol treated heart was partially reversed by the TA extract. Also, the antioxidant response element binding activity of the transcription factor Nrf2 was enhanced by the TA extract in ISO-induced fibrotic rat heart. This study for the first time demonstrates that in ISO-induced fibrotic heart, TA extract improves cardiac function by regulating various gene regulatory modules.

Keywords: Cardiac fibrosis; *Terminalia arjuna*; Isoproterenol; Cardio protection; Antioxidant; Oxidative stress

Abbreviations: AREs: Antioxidative Response Elements; AP 1: Activator Protein-1; βAR: Beta-Adrenergic Receptor; Col: Collagen; CTGF: Connective Tissue Growth Factor; ECM: Extracellular Matrix; GPCRs: G-Protein Coupled Receptors; GSH: Glutathione; ISO: Isoproterenol; MMP: Matrix Metalloproteinase; NFκB: Nuclear Factor-κappa B; Nrf2: Nuclear factor erythroid related factor 2; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; TBARS: Thio barbituric Acid Reactive Substance; TA: *Terminalia arjuna;* TGFβ1: Transforming Growth Factor β1; TFs: Transcription Factors.

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Introduction

Cardiac Fibrosis is one of the leading causes of heart failure. It is characterized by the decrease in microvasculature and disruption of normal myocardial structures; a common feature in heart failure patients [1]. Cardiac Fibrosis hardens the ventricles, impeding cardiac contraction-relaxation and separates cardiomyocytes from the extracellular matrix, impairing electrical coupling [2]. Fibroblasts are the predominant mediators of cardiac fibrosis, although the mechanistic insights and the therapeutic options are very limited [3]. A balance between the synthesis of extracellular matrix and its degradation plays an essential role in the maintenance of the structure of the myocardium. Any alteration in its dynamics can lead to cardiac remodeling and dysfunction. When the synthesis of new collagen by myofibroblasts exceeds the rate of its degradation; total collagen content in heart increases and interstitial fibrosis onsets [4]. Conversion of fibroblasts to myofibroblasts contributes towards increased collagen synthesis and TGF-β1 (a polypeptide cytokine) plays a major role in the proliferation of cardiac

fibroblasts, its conversion to myofibroblasts and generation of extracellular matrix proteins [5]. Nevertheless, cardiac fibrosis is a complex pathological response and role of inflammatory and endothelial cells; other cytokines and chemokines and reactive oxygen species is emerging [6]. Beta-adrenergic receptors (β-ARs), the primary GPCRs in heart, are the critical regulators of cardiac functions under both normal and pathological conditions [7]. Patients suffering from chronic heart failure (CHF) have increased endogenous catecholamine level, resulting in substantial alterations in β-AR signaling [8]. Isoproterenol (ISO) is a non-specific β-adrenergic agonist widely used to study the mechanisms of hypertrophy, heart failure and fibrosis both *in vivo* and *ex vivo* [9,10]. The renin-angiotensin-aldosterone system also has a significant role in pathological cardiac remodeling and failure [11]. Both adrenergic agonists and aldosterone stimulate cardiac fibrosis and expression of MMPs [12]. Taken together, although the importance of fibroblasts in the pathophysiology of heart has long been recognized, only a few therapeutic options are available till date [13]. The worldwide users of traditional medicines are quite large and their efficacies have been systematically studied in India and China over the millennium [14]. A number of traditional Indian and Chinese herbs have ameliorating effects on various types of cardiovascular disorders including fibrosis [15]. Among various Indian herbal preparations for treating cardiac ailments, the commonly used one is the bark extract of the *Terminalia arjuna* (Roxb) tree [16]. Its constituents include triterpenes (arjunic acid, arjunolic acid and arjungenin); triterpene glycosides (arjunetin, arjunoglucosides, arjunoside); polyphenols (catechin, gallocatechin, epigallocatechin, arjunin, arjunone and arjunolone); aglycones; β-sitosterol and terminic acid [17]. Number of clinical trials with small populations of patients showed its beneficial effects against dilated cardiomyopathy, myocardial infarction, angina, ischemic cardiomyopathy, hypercholesterolemia, coronary artery diseases, chronic stable angina and heart failure with no serious side effects [18,19]. Number of laboratories has also investigated the effects of TA bark extracts on cardiac hypertrophy, contractile dysfunction, fibrosis and ventricular pressure in experimental animals with induced cardiotoxicity and has demonstrated that TA decreases oxidative stress, reduces cytokine levels and prevents apoptosis [20-23]. Previously, we had demonstrated that TA bark extract prevents isoproterenol induced oxidative stress and fibrosis but does not increase in heart to body weight ratio in rat [21].

Materials and Methods

Reagents

All drugs and biochemicals were procured from Sigma-Aldrich; USA, unless mentioned otherwise. Radioisotopes [γ-^{32}P] ATP was purchased from BRIT, Hyderabad, India. All chemicals were of analytical or molecular biology grade.

Terminalia arjuna extract

The lyophilized aqueous extract of TA stem bark was a gift from the Dabur Research Foundation, Ghaziabad, India. In brief, fresh stem bark of *Terminalia arjuna* was collected from Uttar Pradesh, India, during September-October. Soon after collection, the water extract was prepared as per standard procedures. The ratio of the crude drug to extract was 20:1. The pharmacognostical evaluation of the sample was done as per Ayurvedic Pharmacopoeia of India (API) and Indian Council of Medical Research (ICMR) publications. A voucher specimen is retained [specimen No-R and D/AYU/STD/003] in R and D of Dabur India Limited. The extract was standardized by High Performance Thin Layer Chromatography (HPTLC) fingerprinting done at the Analytical Development Laboratory, R and D Centre, Dabur Research Foundation. Arjunetin, a marker constituent, was procured from Natural Remedies, Bangalore, India and used as the reference. Six constituents were detected with Rf values 0.08, 0.25, 0.43, 0.65, 0.77 and 0.92. The compound detected at Rf 0.25 was identified as arjunetin by co-chromatography with reference standard [24]. The amount of arjunetin present in the extract is 0.10 ± 0.02% w/w. The quantity of active / marker constituents per dosage unit form is 0.75 mg (ranges from 0.73 mg to 0.77 mg). Complete profiling of the constituents of the extract was also done by LC-MS at the Department of Pharmacology and Phytochemistry, Jamia Hamdard, New Delhi, India.

Experimental animals

The study was approved by the Institutional Animal Ethics Committee, All India Institute of Medical Sciences (AIIMS), New Delhi, India. All animal care and experimental protocols were performed in compliance with the National Institutes of Health (NIH) guidelines for the care and use of the Laboratory Animals (NIH Publication no. 85-23, revised 1996). All animals were procured from Central Animal Research Facility at All India Institute of Medical Sciences, New Delhi, India (regd. No. 10/GO/ERebi/SL/99/CPCSEA). Laboratory bred Wistar male rats (150–200 g, 10–12 weeks) were used for the study and maintained under standard laboratory conditions (temperature; 25 ± 2°C, relative humidity; 50 ± 15% and 12 h dark/12 h light period). Solutions of TA stem bark extract and ISO were freshly prepared from the lyophilized power in double distilled water before use. Animals were randomly allocated into following groups (n=6 rats/group) was as follows: Control Group: Rats were administered normal saline (vehicle, 1 ml/kg, body weight) sc. once daily for 8 and 12 weeks. ISO Group: Rats were administered isoproterenol (ISO, 5 mg/kg, body weight) sc. once daily followed by normal saline (1 ml/kg, body weight) orally once daily for 8 and 12 weeks. ISO+TA Group: Rats were administered with isoproterenol (5 mg/kg, body weight) sc. once daily along with pre-administration of water extract of *T. arjuna* (250 mg/kg, body weight) orally once daily for 8 and 12 weeks. Dose of TA was based on a previous study of our group, which showed antifibrotic effect in rat heart following chronic ISO administration [20]. Rats were weighed and euthanized by overdose of anaesthetic ether at the end of administration period. Body weight was taken at the start and end of specified days. The hearts were carefully excised, dipped in ice cold saline, blotted dry with paper towel and weighed. The ratio of gross heart weight to body weight was taken to assess the hypertrophy associated with myocardial fibrosis. Tissues were frozen in liquid nitrogen, and stored at -80°C until further studies.

Echocardiography

Rats were subjected to echocardiography under anesthesia with

ketamine (50 mg/kg) and xylazine (10 mg/kg) after 14 days, in the supine position with the transducer probe placed gently over the left parasternal position. Heart function was evaluated by a two-dimensional M-mode echocardiography using a 10–11.5 MHz cardiac probe transducer with a high frame rate and a shallow focus (10–25 mm) from the short-axis view at the level of the papillary muscles of the LV using the fully digitized Wipro GE system (Vivid 7 dimension). The left ventricular posterior wall (LVPW) and interventricularseptal (IVS) thickness were recorded in systole and diastole. The LV internal dimension was recorded from the short axis view at the level of the papillary muscle from the trailing edge of the septum to the leading edge of the posterior wall. Captured images of five to nine consecutive heart cycles, were digitally transferred online to a computer and subsequently analyzed by an analyst blinded to the treatment groups. Three representative cycles were analyzed and averaged. LV mass, ejection fraction (EF), fractional shortening (FS) and fractional wall thickness (FWT) were calculated as reported earlier [25].

Quantitative real-time PCR

Total RNA from ventricular cardiomyocytes, was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instruction. The RNA samples were treated with DNaseI and then cDNA was generated by using 1-2 µg of total RNA, oligo(dT) primer, and the MMLV reverse transcriptase in a total volume of 20 µl following the manufacturer's instruction (Epicenter, USA). Real-time PCR was performed by using SYBR Green PCR Master Mix (2X) (Applied Biosystems, USA), cDNA and gene-specific primers (summarized in **Table 1** to amplify various transcripts in Applied Biosystems 7500 real-time PCR system (50°C, 2 min; 95°C, 10 min, 1 cycle; 95°C, 15 s; 60°C 1 min, 40 cycles). Expression levels of candidate genes were normalized to 18S rRNA and were presented as fold changes in treated versus control. The ΔC_T value of 18S did not differ significantly between treatment conditions. The real-time PCR data were analyzed using the relative gene expression, i.e., ($\Delta\Delta C_T$) method as described by manufacturer (Applied Biosystem, USA).

Electrophoretic Mobility-Shift Assay (EMSA)

Nuclear extracts from cardiac ventricle were prepared as described by [26] with little modification. Protein content was measured by Bradford assay using BSA as standard. Double-stranded consensus oligonucleotides summarized in **Table 2** were 5' end labeled with a T4 polynucleotide kinase and [γ-^{32}P] ATP as per manufacturer's instruction (Fermentas, USA). Fifteen micrograms of nuclear protein was pre-incubated for 10 min at ice in binding buffer containing 20mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 1mM EDTA (pH 8.0), 1mM DTT and 3% glycerol in a total volume of 80 µl. For competition studies, the pre-incubation was carried out in the presence of 50X molar excess of nonself-cold oligonucleotide probe as compared to specific radiolabelled probe. Thereafter the incubation of the ^{32}P-labled oligonucleotide probe was continued for 1 hr at ice. The reaction was then stopped by adding 2 µl of gel loading buffer (20% ficoll, 0.2% bromophenol blue and 0.2% xylene cyanol) and the protein-DNA binding complexes were analyzed with native 6% polyacrylamide gel electrophoresis in 0.5X TBE buffer (44.5 mmol/L Tris, 44.5 mmol/L boric acid, and 1 mmol/L EDTA). After electrophoresis was conducted, the gel was vacuum dried and exposed to X-ray film (Kodak, USA) with an intensifying screen at -80°C or phosphorimaging machine (Fujifilm, FLA500, Japan). Binding activity of transcription factors on its respective radiolabelled probe was quantified using ImageJ software (NIH Image J, National Institutes of Health, USA) of scanned film.

Biochemical estimations

Heart samples were thawed and homogenized in 10% w/v ice-cold 0.05 M potassium phosphate buffer (pH 7.4). A part of the homogenate was used for thiobarbituric acid reactive substance estimation [27] as marker of lipid peroxidation (product of oxidative free radical). The remaining part of the homogenate was divided into two parts, and processed for estimation of glutathione (GSH) [28], superoxide dismutase (SOD) [29] and catalase [30] activities as measures of endogenous antioxidants. In order to express the enzyme activity (in per mg protein) protein estimation was done by the Bradford assay [31]. The myocardial collagen content was measured in terms of l-hydroxyproline content, as described previously [32].

Statistics

All quantitative data generated in this study were analyzed using SPSS (version 16.0) statistical package. All values were expressed as mean ± SEM. One-way ANOVA followed by Bonferroni post hoc analysis was done for test of significance. A value of $p<0.05$ was considered statistically significant.

Results

Aq. TA extract normalizes cardiac dysfunctions induced by sustained adrenergic stress

In experimental rats, chronic administration of isoproterenol induces cardiac hypertrophy as an early response and heart failure at a later stage [33]. Chronic administration of isoproterenol is also associated with cardiac fibrosis in interstitial cells [34]. While the molecular mechanism of ISO-induced cardiac hypertrophy is well investigated, that of fibrosis is less understood [35]. To evaluate the beneficial effects of TA extract against cardiac fibrosis associated with late stage heart failure, we first reiterated our earlier observation that TA reverses the early stages of fibrosis in rat heart induced by ISO treatment for 4 weeks [36]. In the present study, acute fibrosis and heart failure was incited by sustained ISO treatment for 8 and 12 weeks. Rats were subjected to chronic treatment of ISO (5 mg/kg, body weight) and the induction of pathological hypertrophy was ascertained by measuring the heart to body weight ratio as summarized in **Table 1**. We observed ~1.4 and 1.7-fold increase in heart to body weight ratios after eight and twelve weeks of treatment respectively. In TA plus ISO treated group, such increase in heart to body weight ratio was significantly attenuated at both time points as shown in **Figure 1A**. To assess the cardiac functions under chronic ISO stress in each rat group, M-mode recordings at the level of papillary muscles were done using a 12-MHz ultrasound probe and 2D-echocardiographic analyses were done to evaluate cardiac structure and functions viz.,

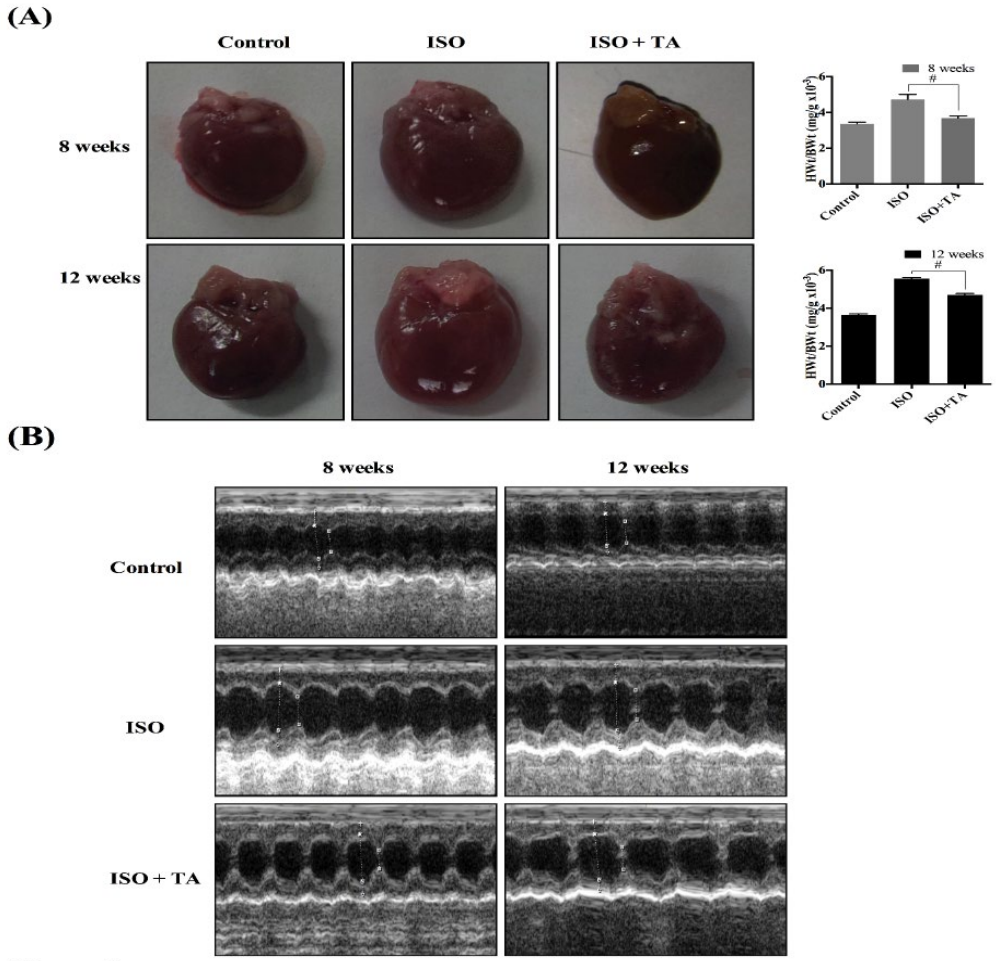

Figure 1 (1A) Aqueous TA extract normalizes heart weight by body weight ratio and collagen content in ISO-induced fibrotic rat heart. Different independent rat groups (n=6) were administered with either ISO alone or pre-administered with aq. TA followed by ISO administration for 8 and 12 weeks. Control rats were administered with saline only. Post-mortem measurements of HWt/BWt (mg/g × 10^{-3}) after 8 and 12 weeks of TA and ISO injection were done to assess cardiac hypertrophy. Right panel is quantitative representation of indicated rat groups under study after TA and ISO administration in terms of HWt/BWt ratio. Data are expressed as mean ± SEM of three independent experiments. The mean difference is significant at 0.05 level. $; p<0.05, *p<0.01, and #; p<0.001 wherever mentioned. (1B) TA extract on M-mode echocardiographic parameters in ISO-induced fibrotic rat heart. Representative echocardiograph of Control, ISO and ISO along with TA group (n=6) for 8 and 12 weeks from three independent experiments.

chamber diameter, wall thickness and left ventricular function. The representative echocardiographs from different rat groups are summarized in **Figure 1B.** ISO-treatment for 8 and 12 weeks showed changes in ventricular geometry causing an increase in IVSd (~1.4 and 1.94-fold), LVPWTd (~1.33 and 1.85-fold), LVIDd (~1.5 and 1.1-fold), LVIDs (~1.2 and 1.16-fold), LV mass (~2.3 and 2.2-fold) and FWT (~2.5 and 2.25-fold) as compared to control. Echocardiography data also demonstrated that ISO infusion for 8 and 12 weeks exhibit decrease in cardiac EF (~5% and 18.5%) and FS (~26% and 34%) as compared to control groups. All these parameters indicated cardiac fibrosis and cardiac dysfunction upon sustained administration of ISO. Aq. TA extract partially restored the changes in these parameters induced by ISO stimulation for 8 and 12 weeks. Details of these echocardiographic parameters in numerical values are summarized in **Table 2**. Thus, TA attenuates cardiac dysfunction in failing heart induced by prolonged adrenergic stress.

Aq. TA extract attenuates oxidative stress by increasing antioxidant enzyme activities and by decreasing lipid peroxidation level

Excessive generation of reactive oxygen species has been attributed to various cardiovascular disorders including fibrosis [37]. However, the mechanisms of generation of ROS under various pathological stages are highly complex and presumably involve multiple mechanisms [38]. Earlier, we have demonstrated that TA extract can ameliorate the fibrotic effects of chronic administration of isoproterenol for 4 weeks [20]. Also, the constituents of TA are quite divergent and widely affect the cardiac proteome and transcriptome [24]. We thus tested whether TA can also mitigate the oxidative stress induced by prolonged administration of ISO. As shown in **Figure 2A-2D**, chronic administration of ISO for 8 and 12 weeks caused increase in myocardial lipid peroxidation by ~3.4 and 4.28-fold respectively as measured in terms of Thiobarbituric Acid Reactive Substance (TBARS). Concomitant decrease in catalase activity by ~0.52 and 0.30-fold, in SOD activity by ~0.43

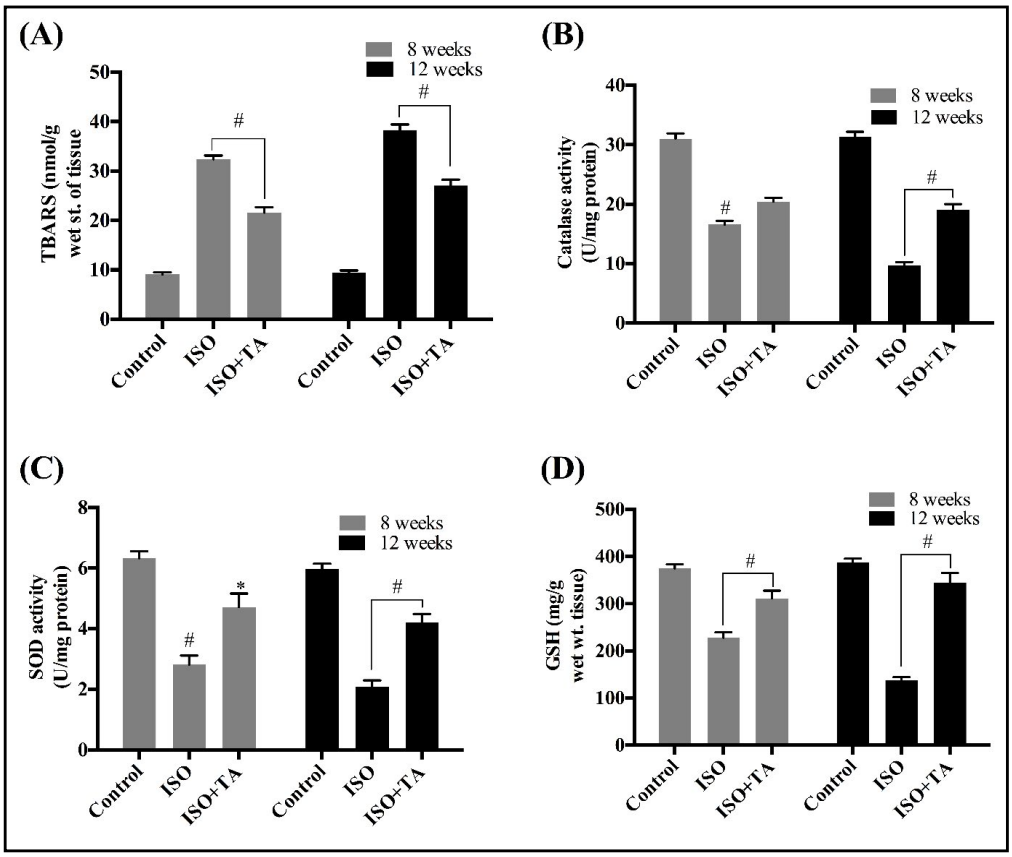

Figure 2 Aqueous TA extract attenuates ISO-induced myocardial fibrosis by regulating various oxidative stress parameters in rat myocardium Different independent rat groups (n=6) were administered with either ISO alone or pre-administered with aq. TA followed by ISO administration for 8 and 12 weeks. Control rats were administered with saline only. Total lysates were prepared from cardiac ventricle of each group to compare various oxidative stress parameters (A) TBARS, (B) catalase activity, (C) SOD activity and (D) GSH content. Data are expressed as mean ± SEM of three independent experiments performed in triplicate. The mean difference is significant at 0.05 level. $; p<0.05 *; p<0.01, and #; p<0.001 wherever mentioned.

and 0.31-fold and in GSH level by ~0.60 and 0.34-fold were also observed. Aqueous TA extract partially restored TBARS level with concurrent increase in catalase, SOD activities and GSH level at 8 and 12 weeks of treatment. Taken together, these data strongly suggest the antioxidant potential of aq. TA extract in ISO-induced failing heart.

Aq. TA extract down regulates profibrotic genes

To determine the extent of fibrosis, l-hydroxyproline content as a marker for heart failure was measured in the ventricular extract. As shown in **Figures 3 and 4,** while collagen content increased substantially by ~2.3 to 4.5-fold respectively for 8 and 12 weeks of ISO treatment, it was significantly attenuated upon co-treatment with aq. TA extract. Interstitial fibrosis can be attributed to either increased collagen synthesis or decreased degradation [2]. To address this aspect, we estimated the transcript levels of collagen 1α and 3α, and that of TGFβ1 and CTGF. Although the upregulation of expression of these genes have been reported in fibrotic heart; the mechanisms of their modulation by chronic adrenergic stimulation and restoration by anti-hypertrophic agents have not been studied [39,40]. As expected, expression of these pro-fibrotic genes increased by ~2.5 to 4.0-fold at 8 weeks and by ~6.5 to 8.0-folds at 12 weeks of ISO infusion and their expression was partially restored by aq. TA extract as shown in **Figure 4A-4D.** Taken together, these data suggest that aq. TA mediates cardioprotection under chronic adrenergic stress by suppressing the activation of key profibrotic genes.

Aq. TA extract regulates the binding activity of various transcription factors associated with ISO-induced fibrotic signaling

Gene regulators play important roles in mediating the induction of hypertrophic and fibrotic genes in heart [41]. To understand whether TA exerts its cardioprotective effects through gene regulatory modules, electrophoretic mobility shift assays were performed with nuclear extracts prepared from the cardiac ventricle of different rat groups under study. Chronic ISO-induced (8 and 12 weeks) fibrotic rat heart showed significant increase in NF-kB (~1.5 to 2.2-fold, **Figure 5A**), AP1 ~2.5 to 4-fold, **Figure 5B** while aq. TA extract restored their expression near control levels. Noticeably, under ISO treatment, while the Nrf2 binding activity decreased by ~0.5 to ~ 0.7-fold (**Figure 5C**) as compared to control, TA extract further increased it. Collectively, our data suggest that aq. TA extract mediates its cardioprotective effects by differentially regulating the DNA binding activities of several transcription factors associated with myocardial fibrosis induced by ISO.

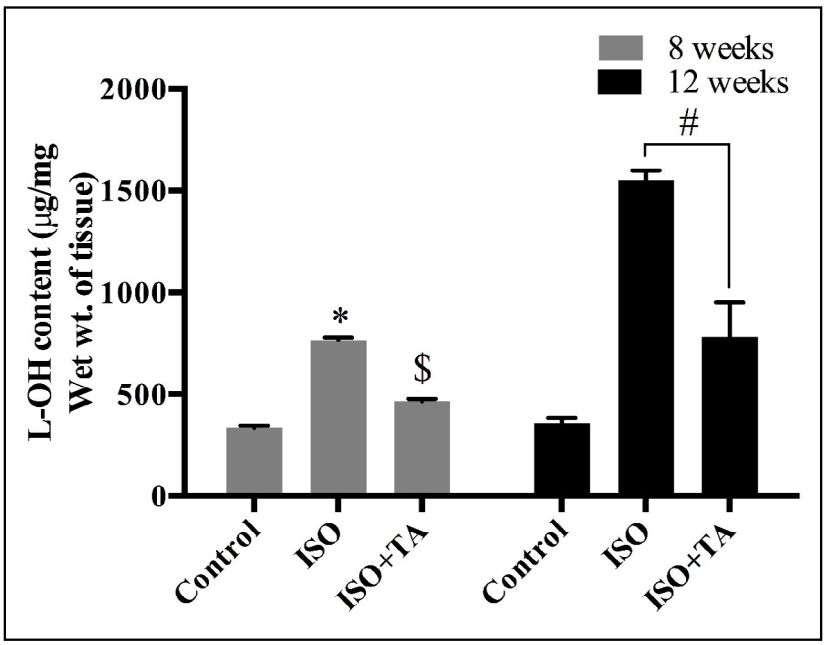

Figure 3 Aqueous TA extract attenuates ISO-induced collagen synthesis in rat myocardium Different independent rat groups (n=6) were administered with either ISO alone or pre-administered with aq. TA followed by with ISO administration for 8 and 12 weeks. Control rats were administered with saline only. Protein from ventricular homogenates of indicated rat groups under study were used to measure extent of fibrosis (collagen content) in terms of l-OH proline. The values shown are mean ± SEM of three independent experiments. The mean difference is significant at 0.05 level. $; p<0.05, *; p<0.01, and #; p<0.001 wherever mentioned.

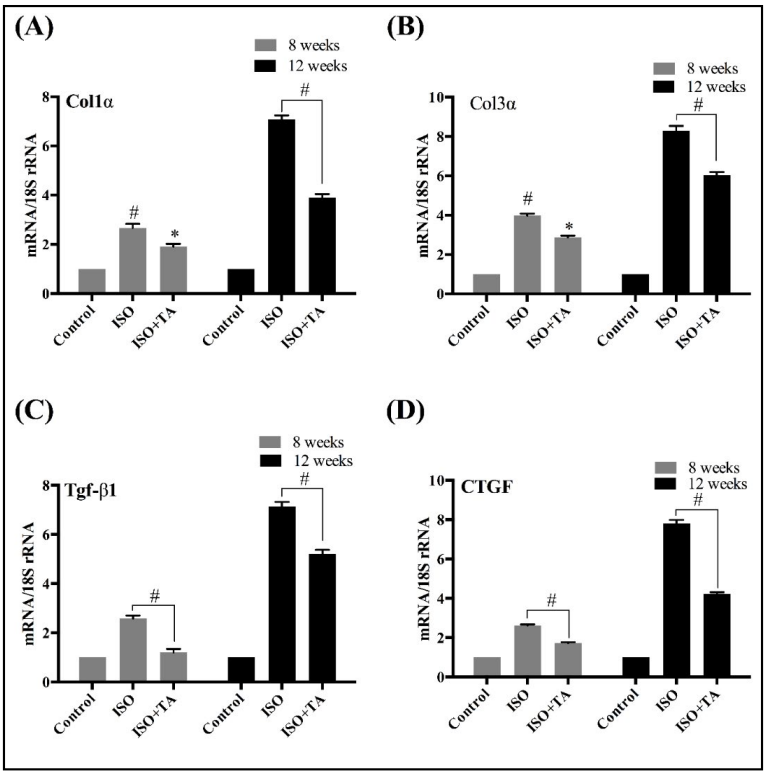

Figure 4 Aqueous TA extract attenuates expression of fibrosis marker genes at transcripts level in ISO-induced fibrotic rat myocardium Different independent rat groups (n=6) were administered with either ISO alone or pre-administered with aq. TA followed by ISO administration for 8 and 12 weeks. Control rats were administered with saline only. RNA from ventricular homogenates of indicated rat groups under study were used for qRT-PCR analyses with gene-specific primers (A) Col1α, (B) Col3α, (C) Tgf-β1, (D) CTGF. 18S rRNA level was used as internal control for normalization of input RNA. Data are expressed as mean ± SEM of three independent experiments performed in triplicate. The mean difference is significant at 0.05 level. $; p<0.05, *; p<0.01, and #; p<0.001 wherever mentioned.

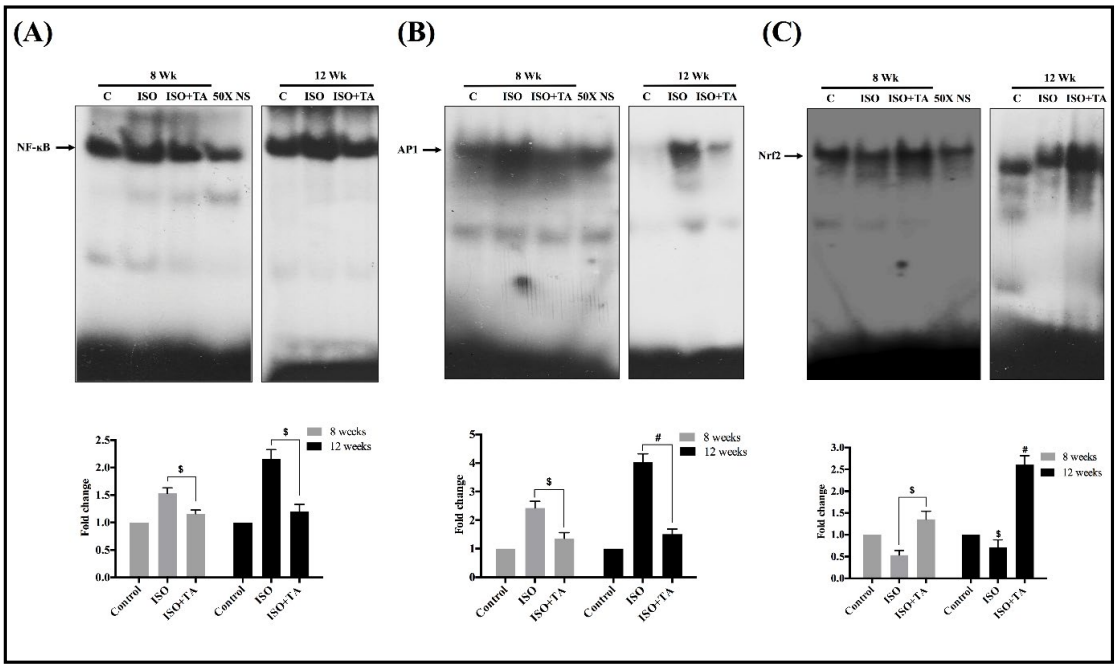

Figure 5 Aqueous TA extract attenuates ISO-induced cardiac fibrosis by regulating the binding activity of different transcription factors Different independent rat groups (n=6) were administered with either ISO alone or pre-administered with aq. TA followed by with ISO administration for 8 and 12 weeks. Control rats were administered with saline only. Equal amount of nuclear extracts (15µg) prepared from left ventricle of each group were incubated with γ^{32}P-radiolabeled probes (A) NF-kB, (B) AP-1, and (C) Nrf2 at 4°C for 1 hr. Competition reactions were performed in presence of 50X molar excess of non-specific (NS) cold oligonucleotide as compared to specific radiolabelled probe. DNA-Protein complexes were then loaded on 6% native poly-acrylamide gel, run at 120 V at room temperature for 2-3 hrs. Representative gels were then dried and representative images were captured by autoradiogram or phosphorimager. Data are expressed as mean ± SEM of three independent experiments. The mean difference is significant at 0.05 level. $; p<0.05*; p<0.01, and #; p<0.001 wherever mentioned.

Table 1 Anatomical data (HWt/BWt) of rat subjected to aqueous TA extract and ISO administration (n=6 in each groups). The mean difference is significant at 0.05 level. #; p<0.001. Compared with the control group.

S. No.	Experimental Groups	(HWt/BWt) × 10^{-3} (Mean ± SEM)
1.	Control-8Wk	3.03 ± 0.096
2.	ISO-8 Wk	4.33 ± 0.275#
3.	ISO+TA-8 Wk	3.6 ± 0.107#
4.	Control-12 Wk	3.8 ± 0.069
5.	ISO-12 Wk	5.46 ± 0.048#
6.	ISO+TA-12 Wk	4.5 ± 0.078#

Discussion

In the present study, we demonstrate that aq. TA extract attenuates cardiac fibrosis and restores cardiac functions in ISO-induced failing heart. Unlike well-defined single entity drugs, TA has multitude of constituents, mode of action(s) of which in isolation is largely unknown [17]. However, when aqueous extract of TA bark is orally administered, it substantially modulates the cardiac proteome and transcriptome [24]. One of the established consequences of ISO administration is the induction of oxidative stress in the heart [42]. However, the mechanisms and the types of ROS generated in heart under norepinephrine stimulation are divergent [38]. It was therefore pertinent to test if TA can also ameliorate the oxidative stress induced by prolonged adrenergic stimulation. As anticipated from the beneficial effects of TA seen with a number of experimental models of cardiovascular disorders [18,20,21,43], aqueous TA extract also mitigated the oxidative stress in rat heart injected with ISO for 8-12 weeks. TA extract effectively reduced lipid peroxidation as assayed by TBARS, and increased the levels of antioxidant enzymes catalase and superoxide dismutase. Since TA is orally administered, it is likely that the active constituents either directly modulate their targets, or they mediate their effects through the metabolites generated from them. Taking into consideration the multiple possible mechanisms of boosting the antioxidant levels, it is too early to infer on the mechanism(s) of action of TA at this stage [44]. In the fibrotic heart, increased collagen deposition produces stiffness, upsetting cardiac systolic and diastolic functions, resulting in heart failure [45]. The antifibrotic effect of TA was also evident from the restoration of cardiac function as evident from the echocardiography that was then corroborated by the downregulation of the mRNA levels of collagen 1α and 3α and that of TGFβ1 and CTGF, the two major extracellular factors that promote fibrosis [46,47]. Taken together, these results for the first time establish that aqueous TA extract prevents cardiac remodeling through the down-regulation of pro-fibrotic cytokines and collagen content by modulating their expression. Since regulation of gene expression in mammalian tissues is a highly complex process involving several regulatory

Table 2 Echocardiography and hemodynamic parameters (Mean ± SEM) of rat heart administered with ISO and aq. TA extract. The mean difference is significant at 0.05 level. $; p<0.05, *; p<0.01, and #; p<0.001. Comparison was done between control vs. ISO group, and ISO vs. ISO+TA group.

Time Point	Parameters	Control (n=6)	ISO (n=6)	ISO+TA (n=6)
8 Wk	IVSd (cm)	0.181 ± 0.005	0.2483 ± 0.008*	0.202 ± 0.006*
	LVPWTd (cm)	0.119 ± 0.02	0.203 ± 0.003$	0.1802 ± 0.003$
	LVIDs (cm)	0.238 ± 0.004	0.283 ± 0.014$	0.249 ± 0.005$
	LVIDd (cm)	0.363 ± 0.02	0.538 ± 0.004#	0.436 ± 0.013$
	FS (%)	57.13 ± 0.905	42 ± 0.74#	55.2 ± 0.608#
	EF (%)	83.7 ± 0.833	79.6 ± 0.796	89.9 ± 0.674#
	LV mass (gm)	0.253 ± 0.015	0.587 ± 0.017#	0.32 ± 0.018#
	FWT	0.519 ± 0.01	1.294 ± 0.083#	0.956 ± 0.061$
12 Wk	**Parameters**	**Control (n=8)**	**ISO (n=8)**	**ISO+TA (n=8)**
	IVSd (cm)	0.136 ± 0.008	0.265 ± 0.002#	0.164 ± 0.007#
	LVPWTd (cm)	0.096 ± 0.003	0.178 ± 0.002#	0.125 ± 0.005*
	LVIDs (cm)	0.255 ± 0.004	0.295 ± 0.013#	0.283 ± 0.002$
	LVIDd (cm)	0.504 ± 0.016	0.55 ± 0.011#	0.532 ± 0.01$
	FS (%)	58.4 ± 1.118	38.9 ± 1.545#	50.5 ± 1.592#
	EF (%)	92 ± 1.27	75.8 ± 1.96#	86.6 ± 1.965#
	LV mass (gm)	0.281 ± 0.014	0.617 ± 0.018#	0.469 ± 0.021#
	FWT	0.4603 ± 0.028	1.037 ± 0.066#	0.505 ± 0.074#

modules, it is difficult at this stage to comment on the precise mode of action of TA, although it is in full agreement with our earlier observation that TA has global effect on the cardiac proteome and transcriptome [24]. To have glimpses of the gene regulatory network mediating the effects of TA, we looked into the modulation of the redox sensitive transcription factors NF-κB, AP-1, and NRF2. During myocardial remodeling, NF-κB induces the activation of cytokines, chemokines, and matrix metallo-proteinases; promoting inflammatory and fibrotic responses [48]. Collagen I and III genes have AP-1 binding sites at their promoters which regulate their expression [49]. We observed that while TA restored the elevated level of NF-κB and AP1 (induced by ISO), it also augmented the Nrf2 activity. It has earlier been reported that the extract from *Terminalia chebula* (also belongs to Combretaceae family like TA) exhibits its anti-inflammatory and antitumor properties through the inhibition of NF-κB-regulated genes [50]. In another study, extract of *Terminalia catappa* mediated anti-metastasis effects through the inhibition of NF-κB and AP1 [51,52]. Our results on the inhibition of NF-κB and AP1 by the TA extract are thus in full consonance with other studies and strongly suggest that TA exerts its effects by suppressing the anti-inflammatory responses mediated by these transcription factors. Nrf2 is a well-studied transcription factor that mediates antioxidant responses [53]. Under normal condition, it is retained in the cytosol by Keap1. In presence of ROS, certain cysteine residues in Keap-1 is oxidized, promoting its dissociation from Nrf2 followed by its translocation into nucleus to bind the antioxidant response elements (AREs) present in the promoter region of numerous genes mediating the antioxidant response [54]. As we observed, ISO repressed the Nrf2 activity below the base line level while TA further enhanced it, suggesting an antioxidant response. Taken together, with this unbiased study (as it is not targeted to a specific regulator or a pathway), we demonstrate the beneficial effect of TA in attenuating cardiac fibrosis caused by chronic ISO stress. Specifically, this is the first report wherein we have used molecular signatures of fibrosis like transcription factors NF-κB, AP-1 and Nrf2 and correlated them with the expression of fibrotic genes in establishing the beneficial effects of TA in rat heart under adrenergic stress. However, further studies will be needed to elucidate the detailed mechanism of its action to enhance its potential therapeutic use against heart failure.

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Conflict of Interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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