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Evidence-based mechanistic role of chrysin towards protection of cardiac hypertrophy and fibrosis in rats

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Abstract

Cardiac hypertrophy is the enlargement of cardiomyocytes in response to persistent release of catecholamine which further leads to cardiac fibrosis. Chrysin, flavonoid from honey, is well known for its multifarious properties like antioxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic. To investigate the cardioprotective potential of chrysin against isoproterenol (ISO), cardiac hypertrophy and fibrosis are induced in rats. Acclimatised male albino Wistar rats were divided into seven groups (n 6): normal (carboxymethyl cellulose at 0·5 % p.o.; as vehicle), hypertrophy control (ISO 3 mg/kg, s.c.), CHY15 + H, CHY30 + H & CHY60 + H (chrysin; p.o.15, 30 and 60 mg/kg respectively + ISO at 3 mg/kg, s.c.), CHY60 (chrysin 60 mg/kg in *per* se) and LST + H (losartan 10 mg/kg p.o. + ISO 3 mg/kg, s.c.) were treated for 28 d. After the dosing schedule on day 29, haemodynamic parameters were recorded, after that blood and heart were excised for biochemical, histological, ultra-structural and molecular evaluations. ISO administration significantly increases heart weight:body weight ratio, pro-oxidants, inflammatory and cardiac injury markers. Further, histopathological, ultra-structural and molecular studies confirmed deteriorative changes due to ISO administration. Pre-treatment with chrysin of 60 mg/kg reversed the ISO-induced damage to myocardium and prevent cardiac hypertrophy and fibrosis through various anti-inflammatory, anti-apoptotic, antioxidant and anti-fibrotic pathways. Data demonstrated that chrysin attenuated myocardial hypertrophy and prevented fibrosis via activation of transforming growth factor-beta (TGF-β)/Smad signalling pathway.

Key words: **Cardiac hypertrophy: Fibrosis: Isoproterenol: Chrysin: TGF-β/Smad2 & Smad3: MAPK/Nrf2-HO-1**

Cardiovascular diseases (CVDs) are major health burden on community and remain undecorated cause of mortality and morbidity worldwide[1]. Though the advances have been made in CVD, it still accounts for 31 % of all deaths worldwide[2]. Cardiac hypertrophy is a compensatory response against stressors such as pressure and volume overload to myocardium which leads to the enlargement of cardiomyocytes[3]. If untreated, it progresses to irreversible damage, which causes an increased myocardial mass, abnormal extracellular matrix deposition and sarcomeric reorganisation[4]. Besides, pathological stimulation of heart results in structural remodelling and activation of various signalling pathways responsible for cardiac hypertrophy. Protein kinase B (Akt/PKB) glycogen synthase kinase-3beta (GSK-3 beta) and transforming growth factor-beta (TGF-β)-mediated Smad-dependent and -independent signalling are various pathways which constitute for cardiac fibrosis and myocardial hypertrophy in response to external stimuli[5,6]. The provocation of fibrosis is evident with oxidative stress, and recruitment of inflammatory cytokines such as Interleukin (IL) and TGF-β signalling proteins was also demonstrated in rats[7,8]. The isoforms of TGF-β promote the phosphorylation cascade of Smad transcription factors (Smad 2/3) which regulates the expression of collagen[9], connective tissue growth factor[10] and matrix metalloproteinases[11]. Balta et al. reported the pro-fibrotic potential of Smad 2/3 in CCl_4-induced liver fibrosis[12], whereas Meng et al. reported the protective effect of Smad 2 over Smad 3 in renal fibrosis[13]. However, the role of TGF-β is controversial for its pro- or anti-fibrotic activity in

Abbreviations: CAT, catalase; CHY, chrysin; CHYP, chrysin per se; CST, Cell Signaling Technology; ERK, extraceullar-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HC, hypertrophy control; HMGB1, high-mobility group box-1; HO-1, haeme oxygenase-1; HSP, heat shock protein-70; ISO, isoproterenol; JNK, c-jun-n terminal kinase; LST, losartan; MAP, mean arterial pressure; MDA, malondialdehyde; Nox-4, nicotinamide adenine dinucleotide phosphate oxidase-4; Nrf-2, nuclear factor erythroid 2-related factor 2; PARP, poly (ADP-ribose) polymerases; SOD, superoxide dismutase; TGF-β, transforming growth factor-beta.

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cardiac fibrosis and myocardial hypertrophy. Furthermore, studies have shown that angiotensin (AT) II by stimulation of AT1 receptor induces various growth factors which led to myocyte proliferation, and hypertrophy and inhibition of AT1 receptor by ARBs have regressed Left Ventricle Pressure (LVH)[14,15]. Previous study by Sim and Chen has demonstrated the anti-cardiac hypertrophic effect of losartan (LST) by inhibition of AT1 receptor[16].

Chrysin (CHY) (5, 7-dihydroxyflavone) is a natural flavonoid obtained from honey, propolis and several fruits and vegetables. It possesses potent anti-inflammatory[17], antioxidant[18], vasodilatory[19] and anti-apoptotic[20] properties. Further, CHY was found cardioprotective in several studies, such as L-NAME-induced hypertension[21], interstitial fibrosis after acute myocardial infarction, when investigated in rats[22], aluminium phosphide-induced cardiomyocyte damage[23] and fructose-induced metabolic diseases[24]. Also, it has been well studied against several models of fibrosis such as chronic kidney disease[25,26], hepatic[24] and lung fibrosis[27]. However, the mechanism of action of its anti-fibrotic property has not been elucidated yet.

Cardiac hypertrophy is characterised with initiation of left ventricular dysfunction which activates compensatory sympathetic activity to restore cardiac output. The sympathetic nervous system provides positive inotropic and chronotropic effects but eventually accelerates the disease progression. Fujita et al. reported the direct correlation between cardiac hypertrophy and increased sympathetic activity in young hypertensive patients[28]. This effect of catecholamines entails both α- and β-adrenergic receptors[29]. The continuous and repeated administration of β-adrenoceptor agonist, isoproterenol (ISO), causes the development of cardiac hypertrophy in few days[30]. On the basis of aforementioned facts, this study was planned to instigate the complex molecular and functional role of TGF-β/Smad signalling in cardiac fibrosis and myocardial hypertrophy by evaluating (1) the effect of CHY in ISO-induced cardiac fibrosis and myocardial hypertrophy, (2) the possible role of TGF-β/Smad signalling behind cardiac remodelling and (3) the interplay between the proposed effects of CHY such as anti-inflammatory, antioxidant, anti-apoptotic and anti-fibrotic.

Material and methods

Drugs and chemicals

ISO, CHY and LST were obtained from Sigma Chemicals Co and Pfizer products, respectively. Creatinine kinase-muscle/brain (CK-MB) isoenzyme and lactate dehydrogenase (LDH) kits were purchased from Elabscience. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (CST 2118), p38 (CST 9212), P-p38 (CST 4631), Bax (CST 2772), Bcl-2 (Abbkine ABM0010), cleaved caspase-3 (CST 9661), cytochrome c (CST 4280), NF-κB (CST 4764), P-NF-κb (CST 3033), extraceullar-regulated kinase (ERK) (CST 4695), P-ERK (CST 4370), c-jun-n terminal kinase (JNK) (ab179461), P-JNK (ab124956), nuclear factor erythroid 2-related factor 2 (Nrf-2) (ab137550), heat shock protein-70 (HSP-70) (CST 4872), haeme oxygenase-1 (HO-1) (ab189491), high-mobility group box-1 (HMGB1) (ab18256), cleaved poly (ADP-ribose) polymerases (PARP) (ITM 3132), TGF-β (CST 3711), nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox-4) (ab109225), Smad 2/3 (CST 5678), p-Smad 2/3 (CST 8828) and β-actin (Abbkine A01010) primary antibodies were procured from Cell Signaling Technology (CST), Abcam (ab), Abbkine and G-Biosciences, respectively. ELISA kits for TNF-α and IL-6 were purchased from Cusabio. All other chemicals used in this study were of analytical grade and were acquired from Sigma Chemicals Co.

Experimental animals

Male albino Wistar rats aged 10–12 weeks (150–200 g) purchased from Institutional Animal House were acclimatised in departmental animal house facility for 7 d prior to the experiment. All the animal experiments on rats were conducted after ethical approval (Approval No. 925/IAEC/16) from Institutional Animal Ethic Committee (IAEC), AIIMS (Registration No. 10/GO/ERebi/SL/99/CPCSEA) following CPCSEA, INSA and ARRIVE guidelines. Animals were housed in polypropylene cages (40 × 25 × 15 cm) in air-conditioned room at 25 ± 2°C and 60 ± 5 % relative humidity in 12:12 light–dark cycle. Animals were fed with chow diet and water *ad libitum*.

Study design

Following acclimatisation, forty-two animals were divided in into seven groups containing six animals in each group. The groups were formed as follows:

Group 1 (N): normal control: 0·5 % carboxymethyl cellulose (CMC); 2 ml/kg/d, p.o.
Group 2 (HC): ISO-control: ISO 3 mg/kg/d, s.c.[5,31]
Group 3–5 (CHY 15/30/60 mg/kg + H): treatment group: CHY (15, 30 & 60 mg/kg/d; p.o.) + ISO 3 mg/kg/d, s.c.[32]
Group 6 (CHYP): *per* se group: CHY 60 mg/kg/d, p.o.
Group 7 (LST + H): standard treatment group: LST 10 mg/kg/d, p.o. + ISO 3 mg/kg/d, s.c.

CHY and ISO were dissolved in 0·5 % of CMC and normal saline, respectively. Animals were administered with 0·5 % of CMC, CHY and LST, and to the respective groups ISO was administered to an interval of 1 h for 28 d. Thereafter, on day 29, animals were anesthetised with pentobarbitone sodium 60 mg/kg, i.p. Surgically, incision was made on the neck and tracheostomy was done to ventilate the animal with positive pressure. Right carotid artery was cannulated and attached to BIOPAC pressure sensing transducers to record various haemodynamic parameters.

Measurement of haemodynamic parameters

The dynamics of blood was measured by recording systolic arterial pressure, mean arterial pressure, diastolic arterial pressure and heart rate using BIOPAC system software (BSL 4.0 MP36). The chest cavity was opened at fifth intercostal space and maintained using retractor. A pressure sensing needle was inserted into the left ventricle to record left ventricular pressures such as left ventricular end-diastolic pressure, left ventricular peak positive and negative pressure (±LV dP/dt).

British Journal of Nutrition

All the recordings were made for 20 min. Thereafter, blood was drawn from heart and serum was separated for the estimation of cardiac injury and inflammatory markers.

Afterwards, animals were killed, heart was excised and weight of whole heart was recorded for the measurement of heart weight:body weight ratio. Thereafter, one part of heart was preserved in 10 % formalin for histopathology, second part was preserved in Karnovsky's fixative for electron microscopy, whereas third part was snap-frozen in liquid N_2 and then stored at −80°C for biochemical and western blot analysis.

Biochemical analysis

Heart tissue was removed from −80°C, and the tissue was weighed and grinded to make 10 % homogenate using ice-chilled phosphate buffer 0·1M, pH 7·4. The homogenate was used to estimate the concentration of malondialdehyde (MDA) and reduced glutathione. The remaining part of the homogenate was centrifuged at 6500 rpm; the supernatant was removed and used for the estimation of enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) and for protein estimation.

Estimation of malondialdehyde level and reduced glutathione content. MDA level in tissue was estimated using protocol given by Ohkawa et al.[33]. To 0·1 ml of tissue homogenate, 990 µl of phosphate buffer (0·1 M; pH 7·4), 1·5 ml of thiobarbituric acid (0·8 %), 0·5 ml of sodium dodecyl sulphate (8·1 %) and 1·5 ml of acetic acid (20 %; pH 3·5) were added. Mixture was heated for 60 min, cooled and subsequently 5 ml of butanol: pyridine (15:1) was added. Organic pink layer was separated, and absorbance was read at 532 nm using microtest plate reader (BioteK). MDA level was calculated from standard linear graph and expressed as nmole/g tissue.

Reduced glutathione content was estimated by protocol given by Moron et al.[34]. Equal volume of homogenate and 10 % tricarboxylic acid was centrifuged at 5000 rpm for 10 min to obtain supernatant. To 50 µl of supernatant, 3 ml of phosphate buffer (M; pH 8·0) and 500 µl of dithiobis (2)-nitro benzoic acid were added and vortexed. Absorbance of resulted yellow colour was recorded at 412 and standard linear graph was plotted. GSH (reduced glutathione) concentration was represented as µmole/g tissue.

Measurement of superoxide dismutase and catalase enzyme activities. SOD activity was measured by method described by Marklund & Marklund[35]. To 100 µl of supernatant, 2·95 ml of phosphate buffer (0·1 M; pH 8·4) and 50 µl of pyrogallol (7·5 mM) were added. Change in absorbance was measured at 420 nm for 2 min at an interval of 60 s. One unit of SOD is defined as the amount of enzyme which is required to produce 50 % inhibition of pyrogallol auto-oxidation under the standard assay conditions and represented as U/mg protein.

CAT enzyme activity was measured by method described by Aebi[36]. To the supernatant, 2 ml of phosphate buffer (50 mM; pH 7·0) and 1·0 ml of hydrogen peroxide (30 mM) were added. Change in absorbance was measured at 340 nm for 30 at an interval of 5 s. One unit of CAT represents 1 µmole of hydrogen peroxide decomposes/min and expressed as U/mg protein. Protein was measured by Bradford method[37] using bovine serum albumin as standard.

Estimation of cardiac injury and inflammatory markers

Serum levels of CK-MB isoenzyme and LDH were estimated for cardiac injury. Whereas, the levels of TNF-α and IL-6 were assessed for inflammation. All these markers were evaluated using kits according to protocol prescribed by manufacturer.

Histopathological analysis

The tissues specimen preserved in 10 % formalin was embedded in paraffin to make blocks. These blocks were cut into thin sections (6-µm thick) with microtome. Sections were fixed on egg albumin-coated slides, deparaffinised, stained with haematoxylin and eosin (H & E), and visualised under light microscope (Dewinter technologies). Also, myocyte cross-sectional area was analysed using FIJI software (ImageJ). The outline of myocytes was traced in each section.

Transmission electron microscopy analysis

The Karnovsky's fixed tissues were processed and embedded in Araldite CY212 to make blocks. Thin sections were cut (approximately 70–80 nm thickness) using an ultra-microtome (Reichert Technologies). Thereafter, the tissues were stained with uranyl acetate and lead acetate. These sections were visualised under a transmission electron microscope (Morgagni 268D; FEI company) operated at 80 kV. The sections were evaluated by a cytologist blinded to the study groups.

Western blot analysis

Tissues preserved at −80°C were used to make 10 % fine homogenate in radio-immune precipitation assay buffer and protease inhibitor. The homogenate was then centrifuged at 10 000 rpm and supernatant was separated. The supernatant was used to estimate the protein concentration using Bradford reagent[37]. The protein concentration equivalent to 40 µg in all samples was mixed with protein loading dye and dilution buffer (TRIS 0·1 M; pH 6·8), loaded on polyacrylamide gel in denaturing and reducing condition (using SDS and β-mercaptoethanol) (SDS-PAGE). The separated proteins were then transferred to nitrocellulose membrane which was then incubated in 3 % bovine serum albumin for 1 h to block the unoccupied site on the nitrocellulose membrane. Thereafter, the membranes were incubated with primary antibody at a dilution of 1:5000 for 8 to 12 h at 4°C, followed by HRP-conjugated secondary antibody at a dilution of 1:5000 for 3 h at room temperature. The bounded antibodies were then visualised with an enhanced chemiluminescence (ECL) (Thermofisher Scientific Inc.) kit and quantified by densitometric analysis. The images of protein expression were assessed using ImageJ software. The densities of the bands of all groups were compared with normal group. Further, the ratio of phosphorylated:dephosphorylated form of the protein was calculated and represented to assess the intergroup variation in the levels of the given protein.

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Statistical analysis

All the data are expressed as mean ± SEM. Results were analysed using one-way ANOVA followed by *post hoc* test (Tukey) using Sigma Plot 12.0 software. *P*-value < 0·05 was considered as statistically significant. Hypertrophy control (HC) and CHY *per* se (CHYP) groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group.

Results

Effect of CHY on heart weight:body weight ratio

ISO administration expressed myocardial hypertrophy or cardiac fibrosis characterised by significantly increased heart weight:body weight ratio. CHY at all doses significantly ($P < 0·001$) blunted the effect of ISO. Further, CHYP and LST group also significantly diminished the ISO-induced hypertrophy (Fig. 1). ISO administration resulted in increased heart weight:body weight ratio and treatment with CHY at all doses significantly lessened this effect.

Effect of CHY on haemodynamic parameters

The recording of systolic arterial pressure, mean arterial pressure and diastolic arterial pressure in normal group was approximately 120/100/85 mmHg, respectively. ISO administration showed significant ($P < 0·001$) decrease in arterial pressure (systolic arterial pressure, mean arterial pressure and diastolic arterial pressure) and increase in heart rate ($P < 0·001$) as compared with normal group. Pre-treatment with CHY 15 and 30 mg/kg group did not show marked improvement in haemodynamic parameters; however, in CHY 60 mg/kg + H group, there was significant ($P < 0·001$) reversal of all systolic arterial pressure, mean arterial pressure, diastolic arterial pressure and heart rate as compared with HC group. These markers were appreciably similar in CHYP and LST group as compared with normal group, and the recordings were significantly ($P < 0·001$) contrasting to that of HC group (Fig. 2(a) and (b)). Further, ISO administration led to ventricular dysfunction evidenced by significant ($P < 0·001$) elevation of left ventricular end-diastolic pressure (pre-load) and decline in ±LV dP/dt (contraction and relaxation) as compared with normal group. These left ventricular pressures were remarkably ($P < 0·001$) improved in 60 mg/kg CHY +H and LST + H group. However, no such effect was seen at CHY15 + H and CHY30 + H groups (Fig. 2(c) and (d)). Furthermore, no significant change in any of the haemodynamic parameter was observed between normal and *per* se group.

Effect of CHY on biochemical parameters

The levels of pro-oxidant (MDA) and antioxidants (GSH) were similar in both normal and CHYP groups. However, lower levels of SOD and CAT were observed in CHYP group. ISO administration showed decreased levels of GSH, SOD and CAT ($P < 0·001$) with a concomitant increase in MDA ($P < 0·001$) levels as compared with normal group. This imbalance between pro- and antioxidant levels were significantly ($P < 0·001$) reversed in CHY60 + H group as compared with HC group. However, the effect of CHY at 15 and 30 mg/kg was not consequential with high dose of CHY (60 mg/kg). Also, these levels were significantly ($P < 0·001$) improved with LST 10 mg/kg (LST + H) as compared with HC group (Table 1). ISO resulted in depletion of antioxidants, whereas CHY at the highest dose (60 mg/kg) significantly bolstered the antioxidant status.

Effect of CHY on cardiac injury markers

ISO administration is accompanied by the formation of lesion in myocardial membrane thereby releasing several cardiac enzymes in serum. The levels of CK-MB and LDH were significantly raised ($P < 0·001$) with ISO administration as compared with normal group. The release of these cardiac markers was significantly ($P < 0·001$) prevented with CHY 60 mg/kg. Though, the levels were improved with the lower doses of CHY as well (CHY15 + H and CHY30 + H), but no statistical significance was found in comparison with HC group. Moreover, in LST group, the levels were significantly

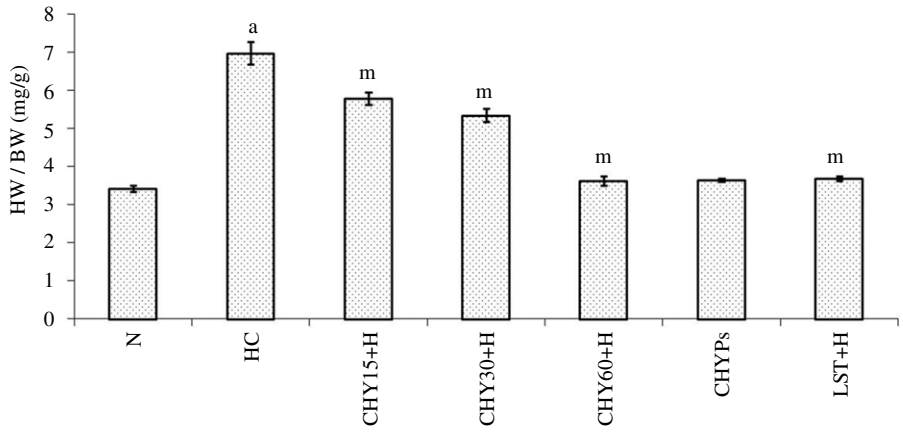

Fig. 1. Effect of CHY on HW:BW ratio. Data are expressed as mean ± SEM; *n* 6 in each group. *P*-value is represented as a: $P < 0·001$ *v.* N group and m: $P < 0·001$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. HW: BW, heart weight:body weight; N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYPs, chrysin *per* se; LST, losartan; H, hypertrophy.

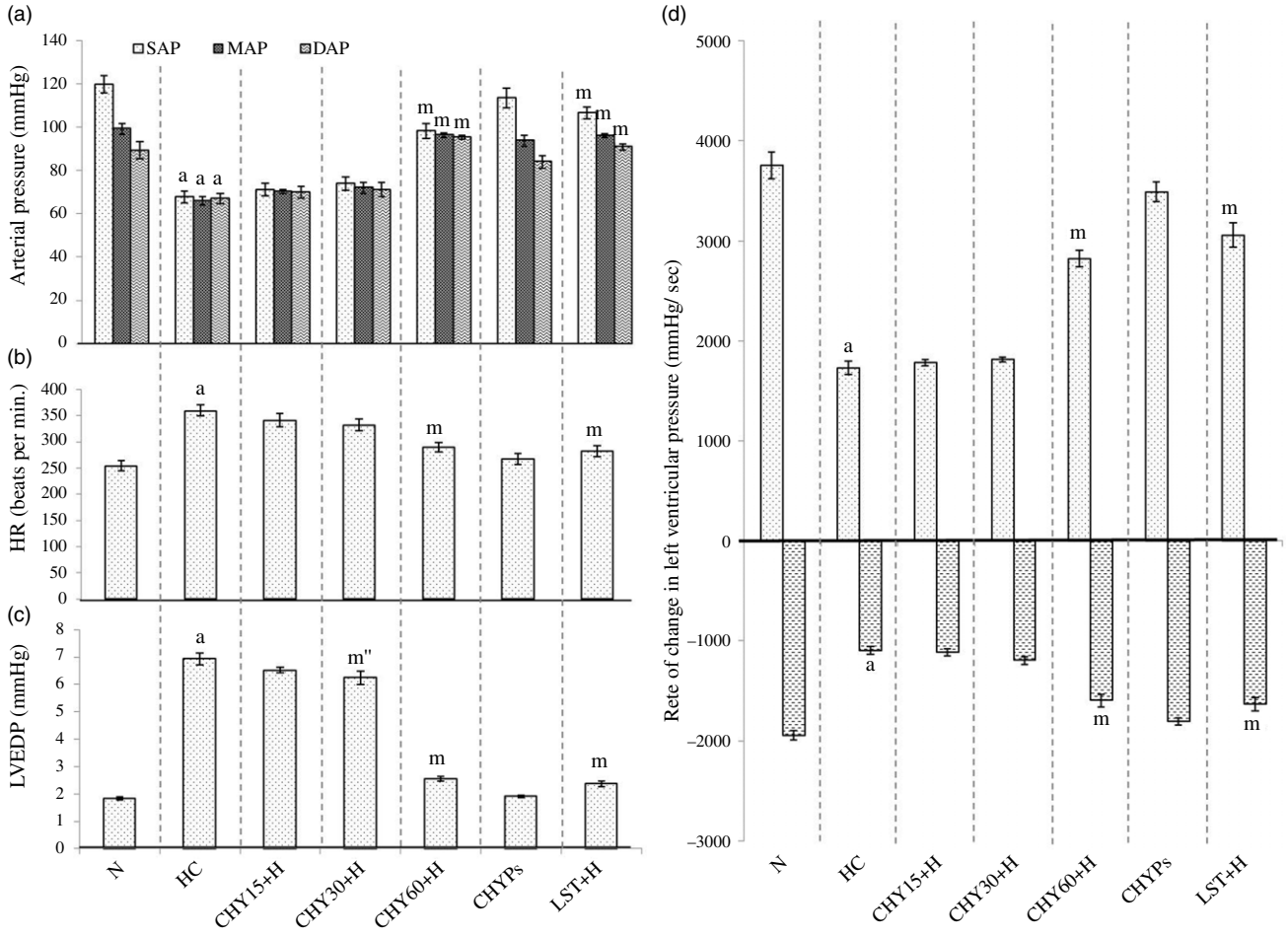

Fig. 2. Effect of CHY on haemodynamic parameters. Data are expressed as mean ± SEM; *n* 6 in each group. *P*-value is represented as a: $P < 0{\cdot}001$ *v.* N group and m: $P < 0{\cdot}001$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. SAP, systolic arterial pressure; MAP, mean arterial pressure; DAP, diastolic arterial pressure; LVDEP, left ventricular end-diastolic pressure; (±) LV dP/dt, rate of change of left ventricular pressure; N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

Table 1. Effect of CHY on biochemical and cardiac injury markers
(Mean values with their standard errors of the mean, *n* 6 in each group)

	MDA (nM/g tissue)		GSH (μg/g tissue)		CAT (U/mg tissue protein)		SOD (U/mg tissue protein)		CK-MB (U/L)		LDH (U/L)	
Groups	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
N	63·41	2·60	2·92	0·11	5·39	0·21	4·84	0·28	92·58	3·70	79·81	2·93
HC	176·29*	6·93	1·60*	0·07	2·32*	0·09	1·37	0·03*	151·84*	3·69	367·18*	14·79
CHY15 + H	171·85	7·01	1·77	0·07	2·67	0·10	1·89	0·22	144·43	4·95	344·22	8·67
CHY30 + H	168·18	6·86	1·90	0·08	2·72	0·05	2·26	0·11	137·02	3·69	336·08	6·69
CHY60 + H	130·65**	5·04	2·45**	0·10	4·59**	0·18	3·98**	0·34	111·1**	1·98	160·17**	5·22
CHYP	77·81	3·14	2·72	0·11	3·94*	0·12	3·15	0·29*	98·51	2·47	157·95*	4·74
LST + H	122·69**	5·01	2·54**	0·10	4·49**	0·14	3·75**	0·04	108·14**	2·84	165·72**	2·50

MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CK-MB, creatinine kinase-muscle/brain; LDH, lactate dehydrogenase; N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy
P-value is represented as *$P < 0{\cdot}001$ *v.* N group; **$P < 0{\cdot}001$ *v.* HC group. HC and CHYP groups are compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group.

($P < 0{\cdot}001$) improved in comparison with HC group (Table 1). However, significant increase in LDH levels was observed in CHYP group. ISO caused increase in level of cardiac injury markers in the serum, and CHY 60 mg/kg significantly inhibited their release and thus reduced their level in the serum.

Effect of CHY on inflammatory markers

ISO administration for 28 d at 3 mg/kg/d significantly ($P < 0{\cdot}001$) promoted the release of TNF-α and IL-6 in serum as compared with normal group. Pre-treatment with CHY 60 mg/kg and

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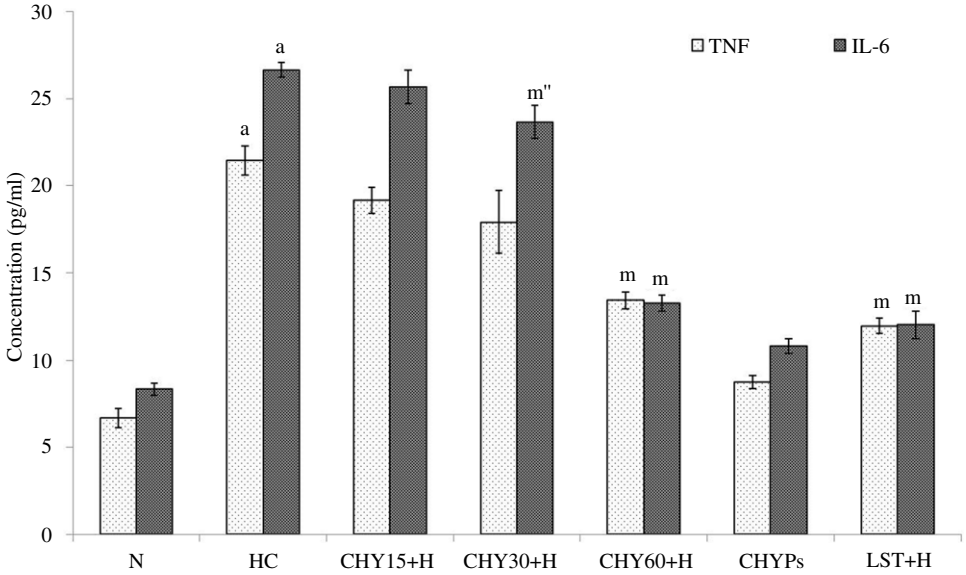

Fig. 3. Effect of CHY on inflammatory markers. Data are expressed as mean ± SEM; *n* 6 in each group. *P*-value is represented as a: $P < 0{\cdot}001$ *v.* N group and m: $P < 0{\cdot}001$, m": $P < 0{\cdot}05$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

LST significantly ($P < 0{\cdot}001$) averted the release of these inflammatory cytokines. The levels of TNF-α and IL-6 were similar in CHYP and normal group. However, no such remarkable reversal was observed at CHY 15 and 30 mg/kg doses (Fig. 3). ISO administration for 28 d resulted in inflammation in the cardiac tissue, whereas highest dose of CHY, that is, 60 mg prevented the inflammation.

Effect of CHY on morphological changes in the tissue specimen

Histopathological examination at 20× magnification showed regular architecture in normal group which was disturbed with continuous ISO administration. The injury was depicted with the presence of inflammation, oedema, increased interstitial spaces, inflammatory cell infiltration and damaged myocardial fibres. The severity of ISO-induced injury was reduced with subsequent increase in the dose of CHY. Though interstitial spaces and infiltration of pro-inflammatory markers were lesser with LST treatment in CHYP group, there was no marked changes occur w.r.t. normal group (Fig. 4(a)). Also, the cardiomyocyte area was significantly ($P < 0{\cdot}001$) elevated in HC group as compared with normal group. The area was significantly ($P < 0{\cdot}001$) reduced at CHY 60 mg/kg and LST 10 mg/kg. However, no such improvement was observed at lower doses of CHY (15 and 30 mg/kg) (Fig. 4(b)). CHY at the dose of 60 mg/kg ameliorated all the pathological changes caused by ISO with lesser effect on lower doses.

Effect of CHY on ultra-structural changes

The electron microscopy revealed myofibrillar disarray and mitochondrial swelling in HC group as compared with normal group. Pre-treatment with CHY 60 mg/kg + H group showed lesser mitochondrial swelling and nuclear condensation. The myocardial architecture was completely preserved in CHYP and LST group comparable to normal group. However, at CHY 15 and 30 mg/kg doses, remarkable myofibrillar damage was observed (Fig. 5). ISO induced morphological changes in cardiac tissue was attenuated by CHY 60 mg/kg dose.

Effect of CHY on several proteins participating in molecular signalling

Samples loaded to the polyacrylamide gel electrophoresis in denaturing and reducing conditions were of in equal protein concentration (Fig. 6(a)). The protein expressions were presented in the 40 µg proteins of tissue homogenates in each group.

Apoptotic signalling. The expression of pro-apoptotic protein GAPDH was increased in HC group as compared with normal group. Its expression was significantly declined in CHY 60 mg/kg + H and LST group as compared with HC group. However, significant increase in GAPDH levels was observed in CHYP group in comparison with normal group (Fig. 6(b) and (c)). The ratio of Bcl-2:Bax is responsible for maintaining the downstream signalling of apoptotic pathway. The ratio was significantly decreased in HC group as compared with normal group, whereas pre-treatment with CHY significantly enhanced this ratio with increasing doses. The Bcl-2:Bax ratio in LST group was similar to normal group (Fig. 6(b) and (d)). Decrease in apoptotic (Bax), increase in anti-apoptotic (Bcl-2) and increase in Bcl-2/Bax were observed in CHYP group (Fig. 6(d)). Further, the expression of downstream signalling proteins, that is, PARP, cytochrome-C and caspase-3 were following the similar pattern (Fig. 6(b) and (e)). The levels of these three proteins were significantly ($P < 0{\cdot}001$) increased in HC group and were further rose in lower doses of CHY (15 and 30 mg/kg); however at CHY 60 mg/kg treatment group, the levels were remarkably ($P < 0{\cdot}001$) reduced. Pre-treatment

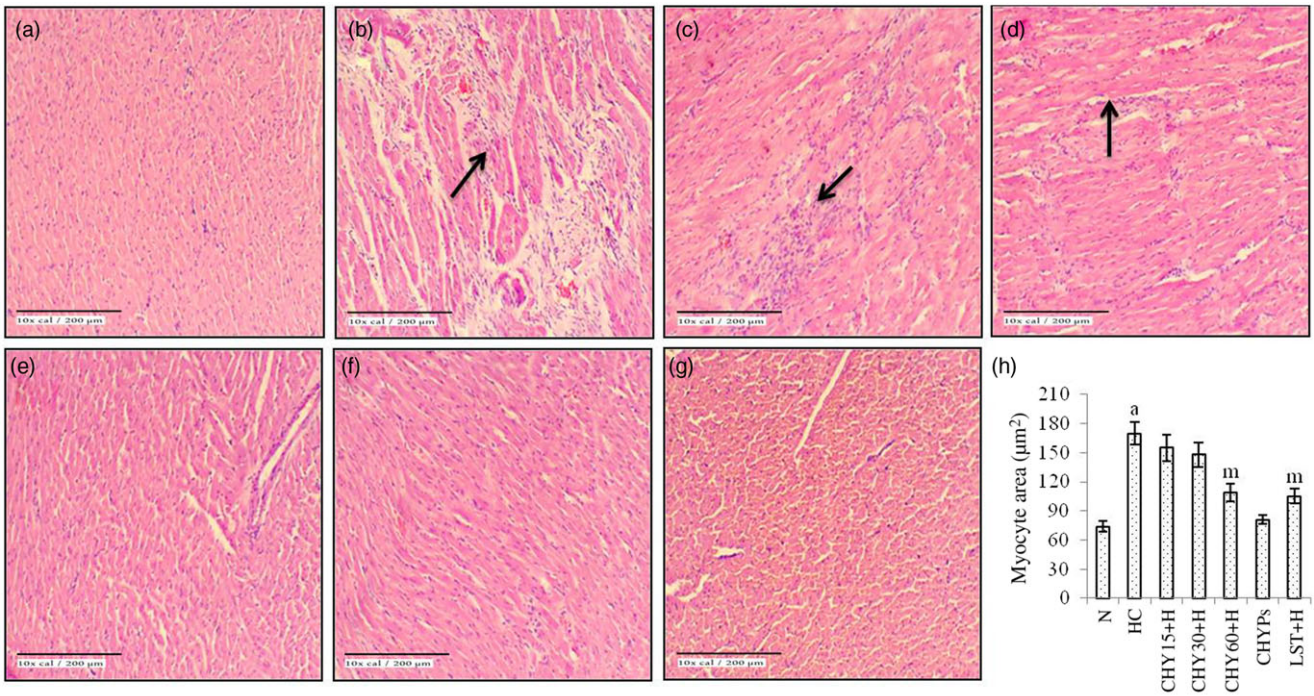

Fig. 4. Effect of CHY on cardiac histopathology (20X; *n* 3; scale bar 50 μm; haematoxylin–eosin staining). A: normal; B: cardiac hypertrophy; C: chrysin 15 mg/kg +H; D: chrysin 30 mg/kg+H; E: chrysin 60 mg/kg+H; F: chrysin *per* se; G: LST 10 mg/kg+H; and H: graph showing difference in myocyte area (μm^2) in between the groups. HC and CHYPs groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. *P*-value is represented as a: $P < 0{\cdot}001$ *v.* N group and m: $P < 0{\cdot}001$ *v.* HC group. Arrow (à) shows inflammation in the tissue.

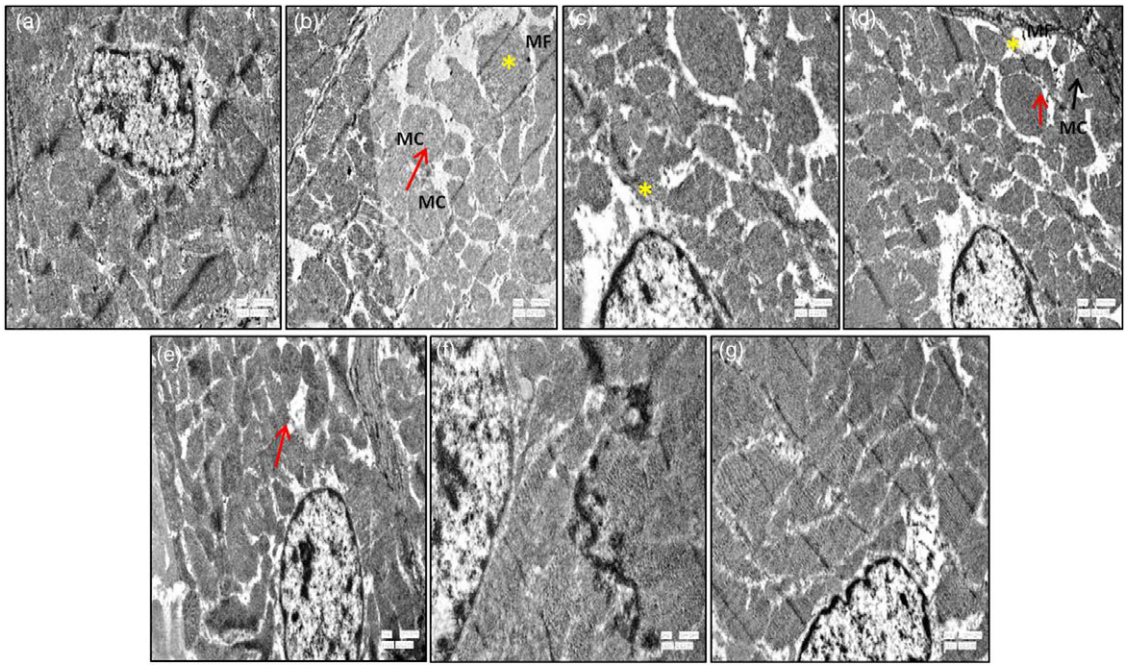

Fig. 5. Effect of CHY on ultra-structural changes. (A–G; scale bar:1 μm). A: normal; B: cardiac hypertrophy; C: chrysin 15mg/kg +H; D: chrysin 30 mg/kg+H; E: chrysin 60 mg/kg+H; F: chrysin *per* se; and G: losartan 10 mg/kg+H. H, hypertrophy; MC, mitochondria; MF, myofibrils. Red arrow (à) indicates mitochondrial damage and yellow star (*) indicates myofibril damage.

with LST showed increased PARP and cytochrome-C, whereas decreased levels of caspase-3. ISO caused apoptosis as marked by increased level of apoptotic proteins (Bax, PARP, cytochrome-C and caspase-3) and decrease in anti-apoptotic protein (Bcl-2) and CHY (60 mg/kg) significantly reversed these apoptotic changes (Fig. 6(b) and (f)).

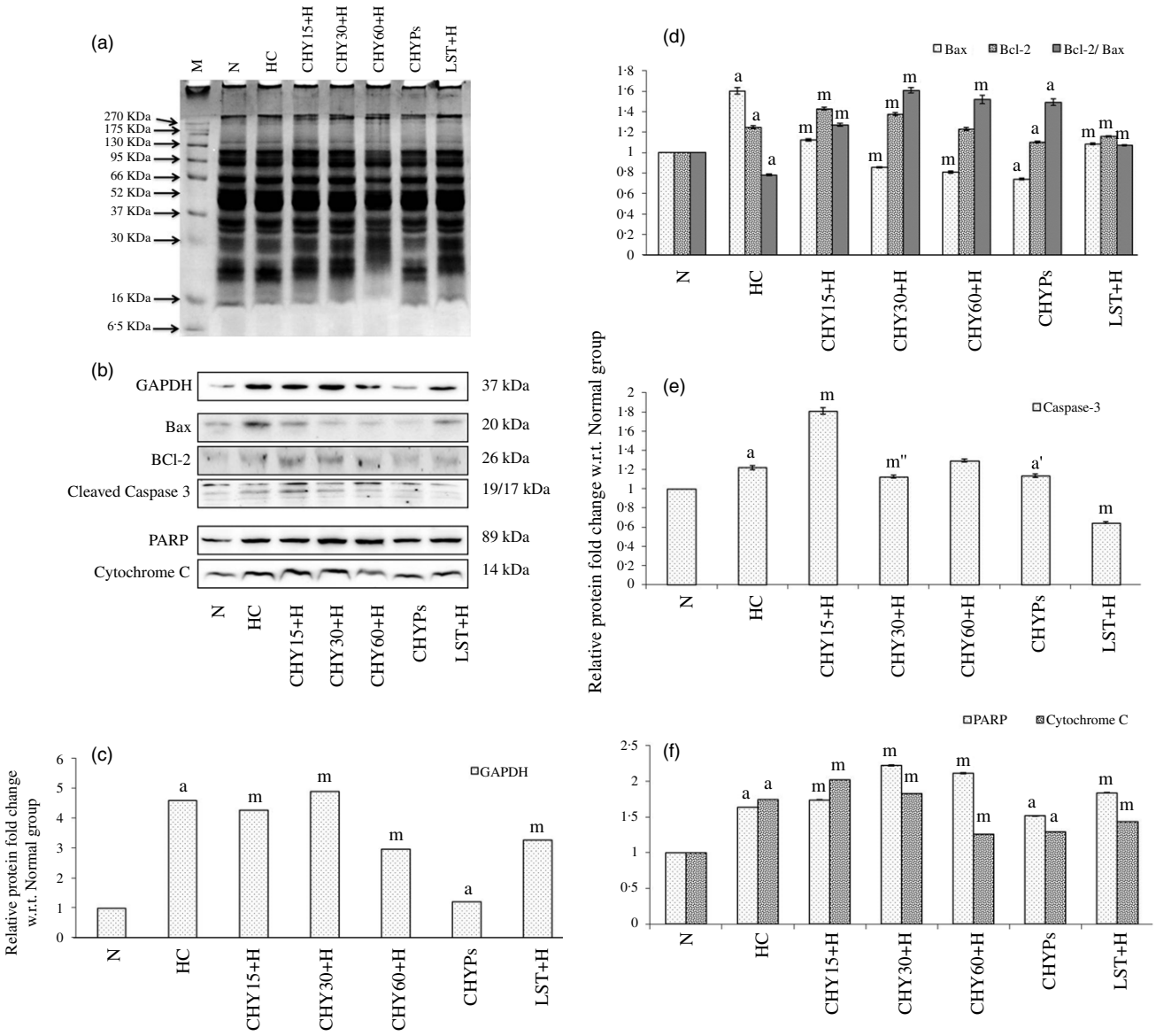

Fig. 6. Effect of CHY on apoptotic protein expressions. A: SDS-PAGE of homogenate (Coomassie-stained); B: immunoblots of GAPDH, Bax, Bcl-2, caspase-3, PARP and cytochrome C; C: GAPDH; D: Bax, Bcl-2 and Bcl-2:Bax ratio; E: caspase-3; and F: PARP and cytochrome C. Data are expressed as mean of relative intensity w.r.t. normal group; *n* 3 in each group. *P*-value is represented as a: $P < 0{\cdot}001$, a': $P < 0{\cdot}01$ *v.* N group; m: $P < 0{\cdot}001$, m": $P < 0{\cdot}05$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

MAPK signalling. The ratio of phosphorylated:dephosphorylated form of MAPK family proteins was assessed. The ratio of P-p38:p38 showed decline in HC ($P < 0{\cdot}1$) group as compared with normal group and was further reduced in CHY60 + H ($P < 0{\cdot}001$). However, CHYP group showed significant ($P < 0{\cdot}001$) increase in p38, and decrease in P-p38 and P-p38:p38 group was observed. The ratio was remarkably reduced in the LST-pretreated group. The ratio of P-ERK:ERK and P-JNK:JNK showed similar pattern where the ratio was increased in HC group as compared with normal group which was decreased in CHY groups in a dose-dependent manner (Fig. 7).

TGF-β/ Smad pathway. The ratio of P-NF-κB:NF-κB (Smad-independent pathway) was significantly rose in HC group as compared with normal group. The levels were decreased with pre-treatment of CHY in a dose dependent manner and were similar to normal group in CHY 60 mg/kg + H, LST + H group. Similar trend was seen with the expressions of high-mobility group protein 1 (HMGB1), where increased levels in HC group were reversed to normal with subsequent doses of CHY. However, the expressions of NF-κB, P-NF-κB, ratio of P-NF-κB:NF-κB and the HMGB were lower than the normal control (Fig. 8).

The levels of TGF-*β* (Smad-dependent pathway) were increased in HC group and were further raised with increasing doses of CHY as compared with normal group. Also, these levels were appreciably high in CHYP and LST + H group. Further the levels of Smad 2 and 3 showed contrasting pattern. The levels of

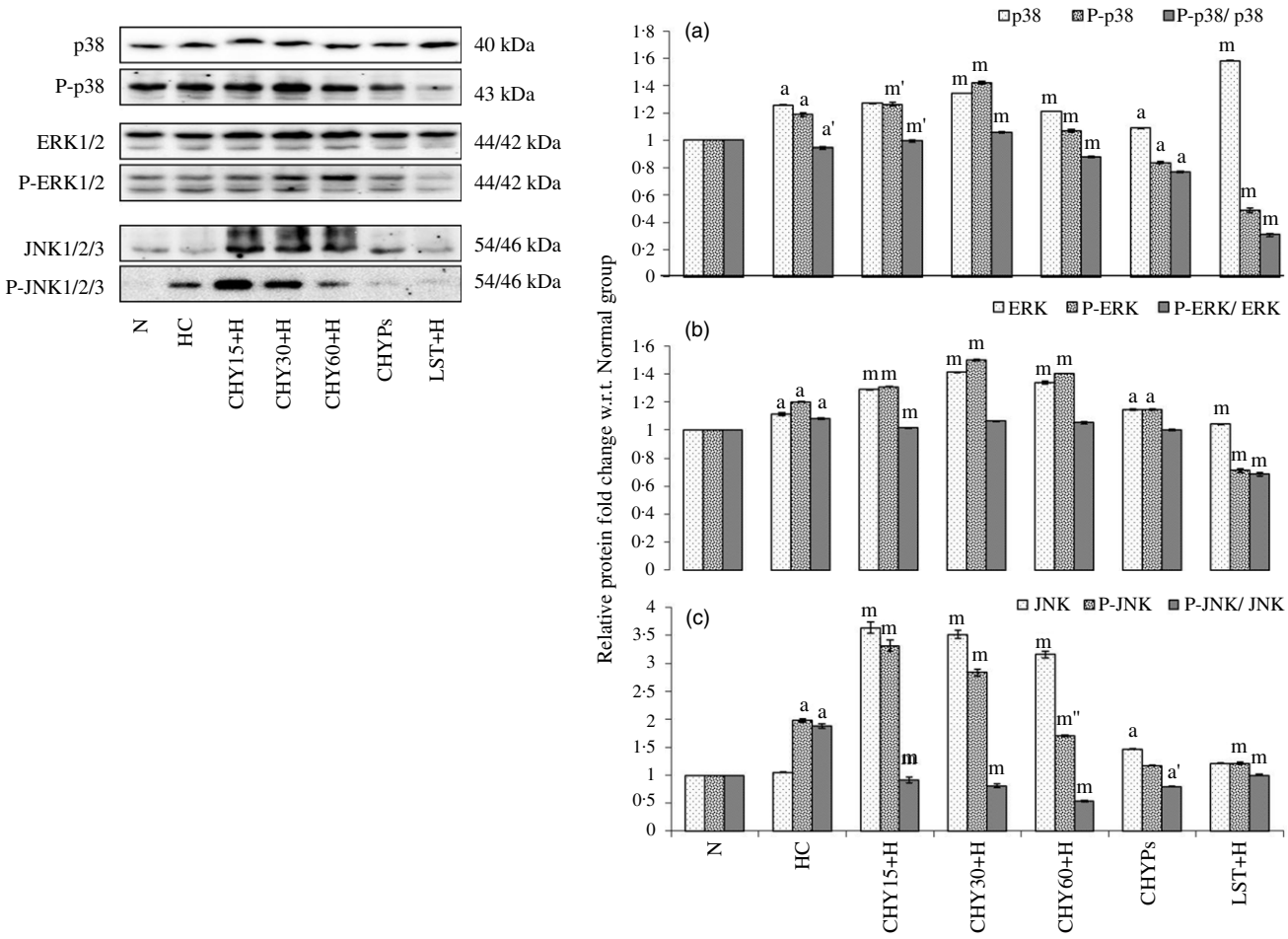

Fig. 7. Effect of CHY on MAPK pathway protein expression. A: p38, P-p38 and P-p38/p38; B: ERK, P-ERK and P-ERK/ERK; and C: JNK, P-JNK and P-JNK/JNK. Data are expressed as mean of relative intensity w.r.t. normal group; *n* 3 in each group. *P*-value is represented as a: $P < 0{\cdot}001$, a': $P < 0{\cdot}01$ *v.* N group; m: $P < 0{\cdot}001$, m': $P < 0{\cdot}01$, m": $P < 0{\cdot}05$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

Smad 3 were increased in HC group as compared with normal group which were decreased and similar to normal group in CHY 60 mg/kg group (CHY60 + H). However, the expressions of Smad 2 and Smad 3 were noticeably increased in CHY 15, 30 and 60 mg/kg groups. The decreased level of Smad 2, increased levels of P-Smad 2 and ratio of P-Smad 2:Smad 2 were observed in CHYP group. However, no changes in Smad 3, P-Smad 3 and P-Smad 3/Smad 3 were observed in CHYP group. Additionally, an appreciably increased ratio of P-Smad 2:Smad 2 to P-Smad 3/Smad 3 was observed at CHY 60 mg/kg (Fig. 9).

Nrf-2/HO-1 signalling. Total expression of Nrf-2 was significantly ($P < 0{\cdot}001$) increased in HC group as compared with normal group which was further increased in CHY60 + H group. The levels were also raised in LST + H group as compared with normal group. The levels of Nox-4 were increased in HC group and were reduced with the increasing doses of CHY as compared with normal group. The level of Nox-4 was declined in LST-pretreated group. The expression of HO-1 was following the similar pattern in CHY 15 and 30 mg/kg group, whereas it was decreased at CHY 60 mg/kg and was similar to normal in LST + H group. Whereas, lower levels of Nox-4 and higher levels of Nrf-2, HO-1 and HSP-70 were observed in CHYP group. The downstream levels of HSP70 followed similar inclination as that of Nrf-2 (Fig. 10).

Discussion

Cardiac remodelling by virtue of intrinsic and external stressors involves compounded response of molecular modifications[38]. Although, the increased overload on myocardium is controlled by our body's compensatory mechanisms such as activation of sympathetic nervous system[28]. Prolonged release of catecholamines leads to irreversible damage to myocardium which is characterised by cellular remodelling followed by fibrosis and cell death[30]. While there is strong evidence that TGF-*β*/Smad proteins negatively regulate cardiac hypertrophy via increased fibrosis[6,39], the molecular mechanism driving this pursuit is hitherto undiscovered. Therefore, well-documented properties of CHY including ant-inflammatory[17], antioxidant[18] and anti-fibrotic potential[12] were appraised against ISO-induced cardiac fibrosis and myocardial hypertrophy model in the present study

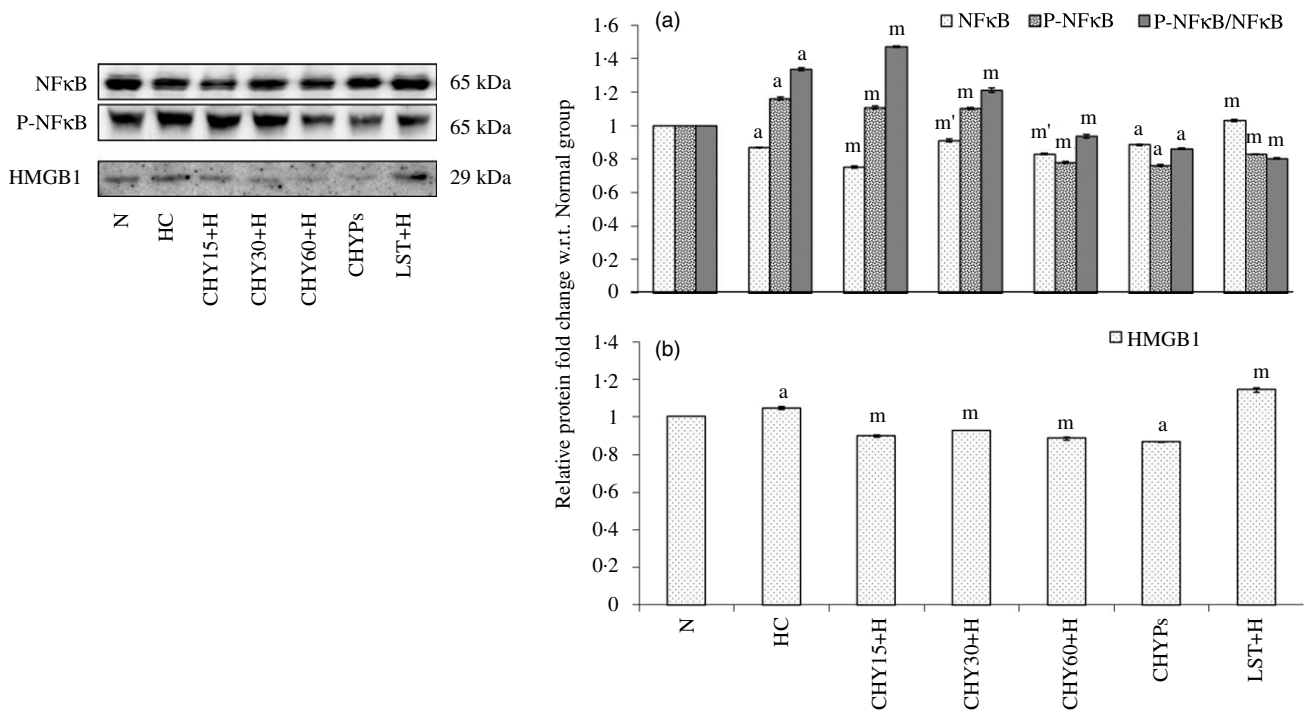

Fig. 8. Effect of CHY on inflammatory marker protein expression. A: NF-κB, P-NF-κB and P-NF-κB/NF-κB, and B: HMGB1. Data are expressed as mean of relative intensity w.r.t. normal group; *n* 3 in each group. *P*-value is represented as a: $P < 0{\cdot}001$ *v.* N group; m: $P < 0{\cdot}001$, m': $P < 0{\cdot}01$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

which was not addressed before. In addition, we accounted for the controversial role of TFG-*β*/Smad along with their interplay with intrinsic stressors pathways such as oxidative stress, inflammation and apoptosis. Accordingly, our findings demonstrated that pre-treatment with CHY 60 mg/kg nullified the injurious effects of continuous ISO administration. Additionally, these effects were comparable to the standard treatment of LST + H 10 mg/kg.

CHY treatment along with the continuous insult of ISO enhanced the cardiac performance with concomitant improvement in arterial pressure, heart rate and left ventricular pressure. These findings were in line with the previous study where CHY improved myocardial injury in diabetic rats[40]. Further, CHY abrogated the ISO-induced cardiac remodelling which was corroborated with decreased heart weight:body weight ratio and pro-oxidant levels, whereas it increased the levels of antioxidants such as GSH, SOD and CAT. In addition, CHY also prevented the disruption of lipid membrane and release of cardiac injury markers as well as inflammatory markers in serum. This sort of cardioprotective role of CHY was previously reported in lipopolysaccharide-induced sepsis[17], doxorubicin-induced cardiomyopathy[41] and cardiotoxicity[42]. The gross effect of continuous ISO administration followed by oxidative stress and release of cardiac markers notably deteriorated the architecture of myocardium which was evinced by increased intracellular spaces, inflammation, oedema, damaged myocardial muscle fibres and presence of huddled nuclei in intercellular spaces due to disrupted cellular membrane. CHY pre-treatment protected the framework of myocardium at 60 mg/kg which was analogous to normal and supercilious to LST group (LST + H). Also, ultra-structural view of the tissue specimen presented a continuous nuclear membrane and normal state of mitochondria which disrupted in HC group due to continuous ISO administration. Similar effect of CHY on histopathology of cardiac myocardium tissues has been reported by Mantawy et al.[42]; however, its effect on ultra-structural level has not been reported before.

Cellular stress initiated on account of oxidative products of ISO, which influences oxidative phosphorylation in the respiratory chain reaction[43]. Following which, mitochondrial membrane permits the release of cytochrome C, an apoptogenic factor which during homoeostasis is averted by maintaining the balance between Bax and Bcl-2. Therewith cytochrome C grabs an energy molecule (ATP) to interact with proteolytic cascade for the activation of caspase-9, which in turn activates caspase-3 and -7[44]. This stream of events disturbs the PARP expression, eventually triggers DNA fragmentation and apoptosis[45]. In present study, we observed the increased expressions of this apoptotic cascade in HC group, whereas pre-treatment with CHY 60 mg/kg blocked the release of cytochrome C by preserving the Bcl-2:Bax ratio. Yet another protein was found which may implicate in this pathway is GAPDH. The profound effect of GAPDH in apoptotic pathway during cardiac hypertrophy has not been admired much. We found that the levels of GAPDH were significantly elevated in the HC group as compared with normal group. The effect was reversed to normal at CHY 60 mg/kg. These findings were in line with previous study who postulated that GAPDH promotes the release of

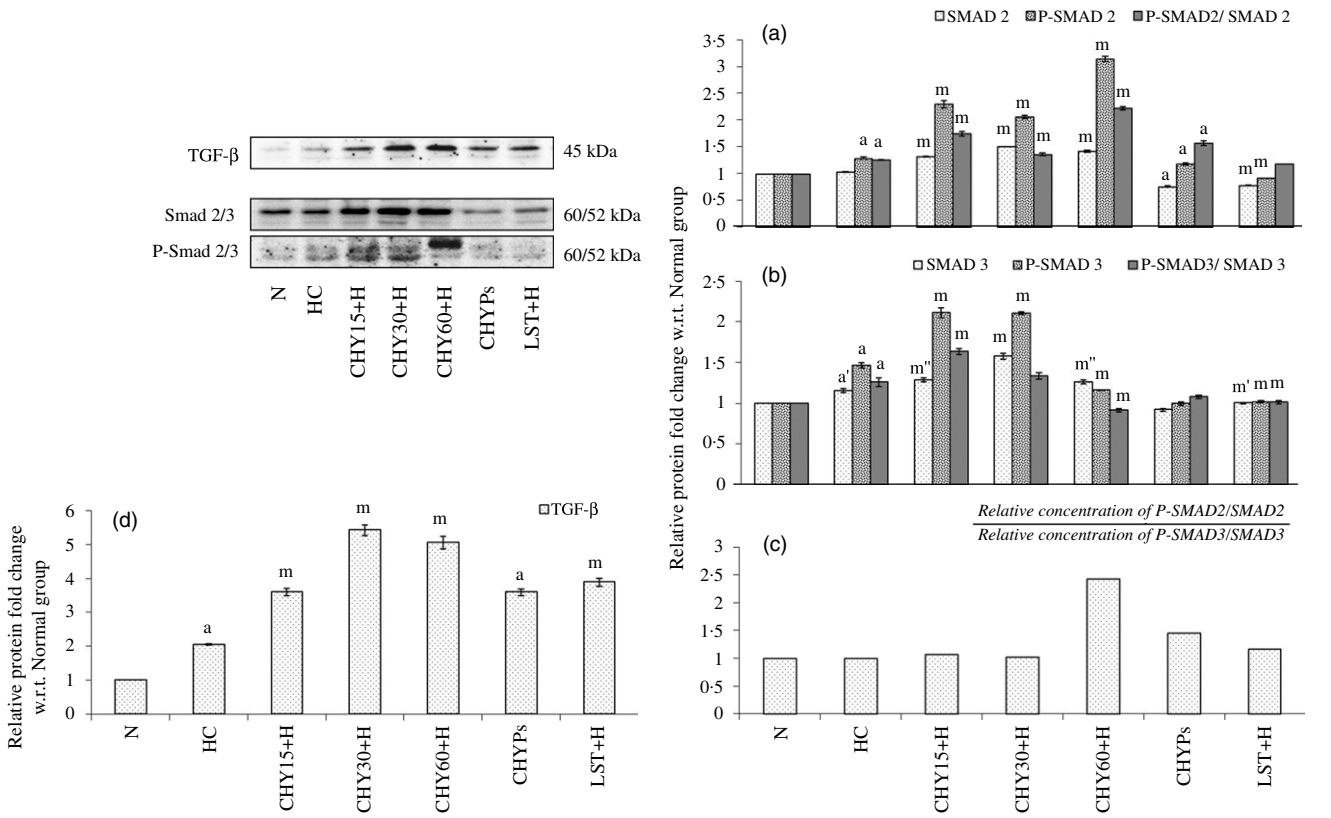

Fig. 9. Effect of CHY on fibrotic proteins expressions. A: SMAD 2, P-SMAD 2 and P-SMAD 2/ SMAD 2; B: SMAD 3, P-SMAD 3 and P-SMAD 3/ SMAD 3; C: P-SMAD 2/SMAD 2 to P-SMAD 3/SMAD 3; and D: TGF-β. Data are expressed as mean of relative intensity w.r.t. normal group; n 3 in each group. P-value is represented as a: $P < 0{\cdot}001$ *v.* N group; m: $P < 0{\cdot}001$, m': $P < 0{\cdot}01$, m": $P < 0{\cdot}05$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

cytochrome C due to nuclear translocation of GAPDH in stressful conditions[46–48]. This could be the possible mechanism behind the apoptotic potential of GAPDH in cardiac fibrosis and myocardial hypertrophy.

Furthermore, it is well evidenced that ISO administration is associated with inflammation and fibrosis through the activation of MAPK and TGF-β signalling[31]. In the present study, the phosphorylated homologues of MAPK family, that is, p38, JNK and ERK1/2 were found elevated in HC group. Also, the expressions of HMGB1, NF-κB followed by IL-6 and TNF-α were raised in HC group. This showed the probable role of inflammation in cardiac fibrosis and myocardial hypertrophy model which was supported by previous studies as well[49,50]. Importantly, the activation of NF-κB is also mediated by HMGB1 via PI3K/Akt pathway orchestrated for inflammation and apoptosis[51–53]. The inflammatory response through MAPK or HMGB1/NF-κB was obstructed with the pre-treatment of CHY 60 mg/kg in our study. Furthermore, the effect of CHY on TGF-β/Smad signalling delineated its potential and role in fibrosis which is a key marker in cardiac fibrosis and myocardial hypertrophy[6]. Previous literature reported that TGF-β is a pro-fibrotic protein which activates its downstream signalling via various isoforms of Smad (2/3) protein[54,55]. We observed an increased expression of TGF-β in ISO-C group which was even more raised with increasing dose of CHY. However, the downstream protein expressions of Smad showed inverse relationship with TGF-β. Additionally, we noticed an opposite relationship in the expressions of Smad 2 and Smad3. This could state that TGF-β activation promotes the levels of both Smad 2 and 3; however, the balance between these two determines the extent of fibrosis. These findings were in line with Meng et al. who reported the novel and protective role of Smad 2 over 3 in fibrosis with two possible mechanisms[13]. First, Smad 2 competes with Smad 3 for its phosphorylation in response to TGF-β, and secondly, the interaction of Smad 2 and 3 with Smad 4 hinders the nuclear translocation of Smad 3 to inhibit its further effects. The findings comprehend the anti-fibrotic effect of CHY via activation of Smad 2 protein. This was also observed in our study due to notably increased ratio of P-Smad 2:Smad 2 to P-Smad 3/Smad 3 in CHY60 + H group as compared with CH.

In the present study, we also found the antioxidant potential of CHY via Nox-4/Nrf2/HO-1 pathway. Nox-4 is generated actively to retaliate against intrinsic or external stressors. Nox-4 and oxidative stress independently potentiate the translocation of Nrf-2 into the nucleus which on tethering with antigen response element generate antioxidants such as HO-1, HSP 70 and NQO-1[56]. Pre-treatment with CHY 60 mg/kg elevated the levels of Nox-4, Nrf-2, HO-1 and HSP 70 which was significantly improved as compared with HC group. This showed the

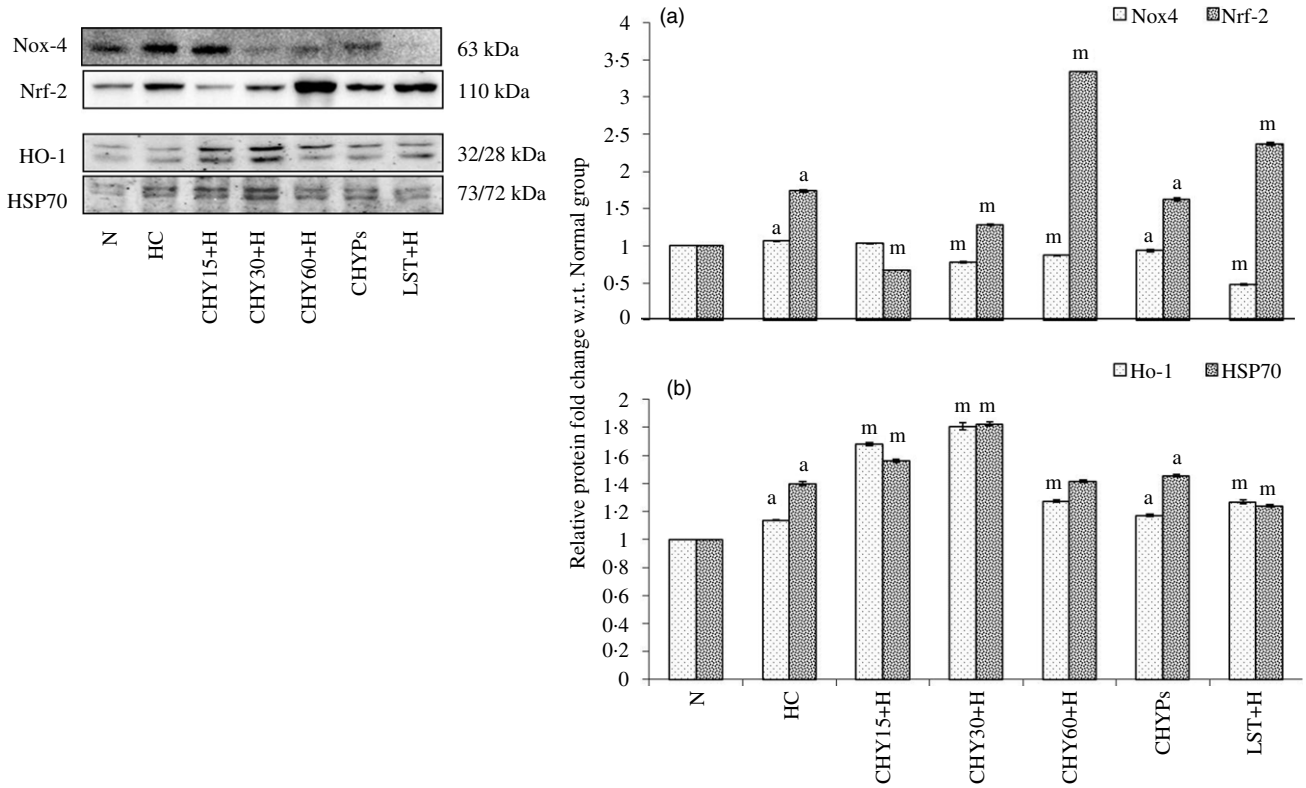

Fig. 10. Effect of CHY on antioxidant pathway protein expression. A: Nox4 and Nrf-2, and B: HO-1 and HSP70. Data are expressed as mean of relative intensity w.r.t. normal group; *n* 3 in each group. *P*-value is represented as a: $P < 0{\cdot}001$ *v.* N group; m: $P < 0{\cdot}001$ *v.* HC group. HC and CHYP groups were compared with N group; however, the CHY15 + H, CHY30 + H, CHY60 + H and LST + H groups were compared with HC group. N, normal; HC, cardiac hypertrophy; CHY, chrysin; CHYP, chrysin *per* se; LST, losartan; H, hypertrophy.

potential antioxidant role of CHY against ISO-induced oxidative damage.

Conclusion

To conclude, the present study highlighted the cardioprotective potential of CHY against ISO-induced cardiac hypertrophy. This can be attributed to its anti-inflammatory (MAPK/NF-κB), antioxidative (Nrf-2/HO-1), anti-apoptotic (Bax/Bcl-2/caspase-3) and anti-fibrotic (TGF-β/Smad 2 and 3) properties. Thus, CHY can be used as a dietary supplement to attenuate the deleterious effects of renin angiotensin-aldosterone system and sympathetic nervous system. However, this is an extensive study which can be correlated clinically towards therapeutic efficacy of CHY. Additionally, the novel roles of GAPDH as pro-apoptotic and Smad 2 as anti-fibrotic proteins have been uncovered in cardiac fibrosis followed by myocardial hypertrophy.

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Authors declare that there is no conflict of interest.

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