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Beneficial Effect of Silymarin in Pressure Overload Induced Experimental Cardiac Hypertrophy

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Abstract

The present investigation was undertaken to study the effect of silymarin on cardiac hypertrophy induced by partial abdominal aortic constriction (PAAC) in Wistar rats. Silymarin was administered for 9 weeks at the end of which we evaluated hypertrophic, hemodynamic, non-specific cardiac markers, oxidative stress parameters, and determined mitochondrial DNA concentration. Hypertrophic control animals exhibited cardiac hypertrophy, altered hemodynamics, oxidative stress, and decreased mitochondrial DNA (mtDNA) concentration. Treatment with silymarin prevented cardiac hypertrophy, improved hemodynamic functions, prevented oxidative stress and increased mitochondrial DNA concentration. Docking studies revealed that silymarin produces maximum docking score with mitogen-activated protein kinases (MAPK) p38 as compared to other relevant proteins docked. Moreover, PAAC-control rats exhibited significantly increased expression of MAPK p38β mRNA levels which were significantly decreased by the treatment of silymarin. Our data suggest that silymarin produces beneficial effects on cardiac hypertrophy which are likely to be mediated through inhibition of MAPK p38β.

Keywords Partial abdominal aortic constriction (PAAC) · Cardiac hypertrophy · Silymarin · MAPK p38 β · Mitochondrial DNA

Abbreviations

MABP	Mean arterial blood pressure
MAPK	Mitogen-activated protein kinases
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
MKK3/6	Mitogen-activated protein kinase kinase 3/6
PAAC	Partial abdominal aortic constriction
CON	Sham control
COS50	Sham control animals treated with silymarin (50 mg/kg/day, p.o)
COS100	Sham control animals treated with silymarin (100 mg/kg/day, p.o)
HYP	Hypertrophic control
HYS50	Hypertrophic animals treated with silymarin (50 mg/kg/day, p.o)
HYS100	Hypertrophic animals treated with silymarin (100 mg/kg/day, p.o)
CRP	C-reactive protein
LDH	Lactate de-hydrogenase
CK	Creatinine kinase
dp/dtmax	Rate of pressure development
dp/dtmin	Rate of pressure decay
CHI	Cardiac hypertrophic index
LVHI	Left ventricular hypertrophic index
LVW/RVW	Left ventricular weight-to-right ventricular weight ratio
HW/BW	Heart weight-to-body weight ratio
MDA	Malondialdehyde
GSH	Reduced glutathione
SOD	Superoxide dismutase
mtDNA	Mitochondrial DNA
GOLD	Genetic optimization for ligand docking
JNK1/2/3	c-Jun NH2 terminal kinases
ERK1/2	Extracellular signal-regulated kinases
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme
PDB	Protein data bank

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Introduction

Cardiac hypertrophy, which represents an adaptive response, is associated with a significant increase in the risk of progression to heart failure [14]. It is affected by several factors like obesity, sedentary lifestyles and diabetes mellitus [18, 37]. However, currently no specific drugs are available for the treatment of cardiac hypertrophy and hence identification of drugs which acts on specific novel targets is the need of the hour. Conventionally, research has focused on identification of pathways of pathological hypertrophy, cardiac dysfunction, and heart failure [32, 38, 43].

Mitogen-activated protein kinases (MAPK) are one of the most prominent regulators of cardiac hypertrophy consisting of a sequence of successive functioning and signalling pathways that ultimately results in the dual phosphorylation and activation of MAPK p38, MAPK c-JNK and MAPK-ERKs. Among all the signalling pathways MAPK p38 is one of the potential targets for induction of pathological cardiac hypertrophy [49]. Out of the two isoforms of MAPK p38 i.e. α and β, p38β isoform promotes greater hypertrophic response than p38α isoform, which tends to promote an apoptotic phenotype [55, 57]. Furthermore, in heart, different extrinsic and intrinsic stimuli leads to activation of mitogen-activated protein kinase kinase kinase 5 (MAP3K5). MAP3K5 activates mitogen-activated protein kinase kinase 3/6 (MKK3/6) which in turn results in activation of MAPK p38β. MAPK p38β triggers different transcription factors which produce alteration of physiology of cardiac myocytes including hypertrophy [47]. Thus, targeting MAPK p38β appears to be an interesting approach for treatment of cardiac hypertrophy.

Milk thistle (*Silybum marianum* L. Gaertn) is a widely used functional food as a liver tonic and hepatoprotective agent. Silymarin, a notable and unique flavonoid complex containing silybin, silydianin and silychrisin, is derived from the milk thistle. Various marketed products of silymarin are available alone or in combination with other chemical constituents for hepatoprotective effect. Silymarin is also reported to prevent lipid peroxidation, inhibit low-density lipoprotein oxidation and scavenge reactive oxygen species [41]. Silibinin, which is one of the major constituents of Silymarin, is reported to exhibit cardioprotective effect in cardiomyocyte [59]. Silymarin is reported to exhibit cardioprotective activity in ischemia reperfusion injury model of rat Rao and Viswanath [44]. It is also reported to exhibit positive ionotropic effect on rat perfused heart [15] and also exhibit cytoprotective effect on cardiomyocytes [54] and plays a role in cardiac preconditioning [58]. However, direct reports of effect of silymarin on cardiac hypertrophy are not available. Hence, we thought it worthwhile to investigate the effect of silymarin on pressure overload model of cardiac hypertrophy. Additionally, silymarin and silibinin are reported to act through MAPK pathway in cancer cell lines [9, 25]. In lieu of this, we speculate that the effect of silymarin on cardiac hypertrophy may be mediated through MAPK.

Methods

The approval of the protocol was taken from the animal ethics committee of the institute (Protocol no: IP/PCOL/MPH/13-1/004, dated—19th Aug, 2013).

Induction of Cardiac Hypertrophy by Partial Abdominal Aortic Constriction (PAAC)

Adult Wistar rats of either sex weighing 250–350 g were maintained under well-controlled conditions of temperature (22 ± 2 °C), humidity (55 ± 5%) and 12/12 h-light–dark cycle. Standard laboratory rat chow and UV-filtered water was provided ad libitum. The rats were randomly divided into six groups each containing 12 rats, CON—Sham control, COS50—Sham control animals treated with silymarin (50 mg/kg/day, p.o), COS100—Sham control animals treated with silymarin (100 mg/kg/day, p.o), HYP—Hypertrophic control, HYS50—Hypertrophic animals treated with silymarin (50 mg/kg/day, p.o), HYS100—Hypertrophic animals treated with silymarin (100 mg/kg/day, p.o).

Pressure overload-induced cardiac hypertrophy was induced by PAAC in Wistar rats carried out under surgical anaesthesia. Surgical procedures were done on third day in PAAC control and PAAC-treated animal under anaesthesia with ketamine (100 mg/kg i.m.) and xylazine (10 mg/kg i.m.) as per earlier reported method [34]. Silymarin was suspended in 0.2% solution of carboxy methyl cellulose and was administered orally from 0th day in sham-treated and PAAC-treated animals and continued up to 9 weeks.

Blood Sample Collection and Serum Analysis

At the end of 9 weeks, blood samples were collected from the retro orbital plexuses under light ether anaesthesia and serum was separated. Serum samples were analysed for total cholesterol, HDL-cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, C-reactive protein (CRP), lactate de-hydrogenase (LDH) and creatinine kinase (CK) by biochemical analyzer (Prietest TOUCH Biochemistry Analyzer, 2.622 A, ROBONIK Pvt. Ltd.) using respective biochemical diagnostic kits (Labcare Diagnostics Pvt. Ltd., India) [19, 22].

Assessment of Hemodynamic Parameters

The animals were anaesthetized by ketamine (100 mg/kg, i.m.) + xylazine (10 mg/kg, i.m.). The body temperature was maintained at 37 ± 1 °C during the experiment. Rat hair was removed from chest and thigh region and the electrodes were inserted. All electrodes were attached to a transducer and ECG signal was recorded using Labscribe Systems (IWORX, New Hampshire, USA). Following this, the electrodes were removed and then carotid artery behind the trachea was exposed and carotid artery was cannulated for the measurement of hemodynamic parameters using a transducer (BP 100) [21, 33, 39, 40]. The hemodynamic parameters observed were mean arterial blood pressure (MABP), heart rate, rate of pressure development (dp/dtmax) and rate of pressure decay (dp/dtmin). All the data were analysed using Labscribe software (Version 118).

Assessment of Hypertrophic Parameters

After measurement of hemodynamic parameters, animals were sacrificed. Heart and femur were isolated from the body. Cardiac hypertrophic index (CHI) is calculated as ratio of heart weight to femur length and left ventricular hypertrophic index (LVHI) was calculated as left ventricular weight-to-heart weight ratio; left ventricular weight-to-right ventricular weight (LVW/RVW) ratio, heart weight-to-body weight ratio (HW/BW) and left ventricular wall thickness were determined [20, 35, 36, 45].

Assessment of Cardiovascular Parameters

Left ventricle (LV) was isolated and homogenized and subjected to estimation of Na^+K^+ATPase activity [53] and collagen levels [42]. Oxidative stress markers like malondialdehyde (MDA), reduced glutathione levels (GSH) and superoxide dismutase levels (SOD) were measured in the LV [17, 39]. Mitochondrial DNA (mtDNA) content was determined by Hoechst assay [4]. Briefly, the mtDNA from LV homogenate was extracted sequentially using chloroform-isoamylalcohol mixture (49:1), sodium acetate, 2-propanol and ethanol. An enriched fraction of mtDNA was determined in a small aliquot with high sensitivity by measuring the fluorescence after binding of the Hoechst 33258 dye.

Histopathological Analysis

A portion of LV was also subjected to histopathological analysis. Hematoxylin and Eosin were used for staining the tissue and the microsections were studied under OLYMPUS (trinocular-CX21FS1) microscope with ×400 magnifications. The cardiomyocyte diameter measurements were taken with Image J analyzer software (NIH) 1.45.

mRNA Expression Studies

MAPK p38β mRNA levels were determined. Briefly, 200–300 mg of heart specimen was taken and homogenized, then total ribonucleic acid (RNA) was extracted using FastRNA® Pro Green Kit (M.P biomedicals) following the instructions of the users' manual. First strand cDNA was synthesized from 1 µg of RNA using First Strand cDNA Synthesis Kit (Novagen) and incubated at 42 °C for 60 min followed by 70 °C for 10 min. PCR was carried out to check gene expression using primers specific for rat β actin (Forward: 5′-CAA CTGGGACGATATGGAGAAG-3′; Reverse: 5′-CTCGAA GTCTAGGGCAACATAG-3) and p38β (Forward: 5′-CAG AAGGTGGCTGTGAAGAA-3′; Reverse: 5′GATGTCCAC TGTCTGGTTGTAG-3′) according to the user manual. The quantification of gene expression was done by Phroetix 1D v 11.4:1DPRO-J3K9-RG5Z-AN (Total lab software).

Docking Studies

Genetic optimization for ligand docking (GOLD 5.2), which is a genetic algorithm-based docking programme, was used for molecular docking. The co-crystallized structure of all the different targets (on which docking of silymarin was performed) was obtained from protein data bank (PDB). Different targets were docked with silymarin. Gold docking results were calculated by considering the gold score fitness, protein–ligand van der Waals and protein–ligand hydrogen bond energy (external H-bond), and higher the GOLD fitness score was considered as better the docked interaction of the complexes. Since the docking score was maximum with MAPK p38, co-crystallized structure of MAPK p38 [PDB id: 3GP0] was taken from PDB and modified for docking calculations [13]. Co-crystallized ligand was removed from the structure; water molecules were removed; H atoms were added, and side chains were fixed during protein preparation. Docking score and number of hydrogen bonds were determined.

Statistical Analysis

All the values were expressed as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-test, using Prism 6.01 (GraphPad Software). A significance level of $p < 0.05$ was considered to be statistically significant.

Results

Hypertrophic Parameters

PAAC control rats exhibited significant increase in CHI, LVHI, HW/BW ratio, LVW/RVW ratio, LV wall thickness,

LV collagen levels and cardiomyocyte diameter as compared to normal control rats. Treatment with silymarin significantly reduced CHI, LVHI, HW/BW ratio, LVW/RVW ratio, LV wall thickness, LV collagen levels and cardiomyocyte diameter in PAAC treated rats as compared to PAAC control rats (Fig. 1).

Hemodynamic Parameters

There was a significant increase in the MABP and a significant decrease in heart rate in PAAC control rats as compared to normal control rats. Treatment with silymarin significantly prevented the increase in MABP and decrease in heart rate in PAAC treated rats as compared to PAAC control rats (Table 1).

The PAAC control rats also exhibited a significantly decreased rate of pressure development and decay as compared to control rats. Treatment with silymarin significantly increased the rate of pressure development and decay in PAAC treated rats as compared to PAAC control rats (Table 1).

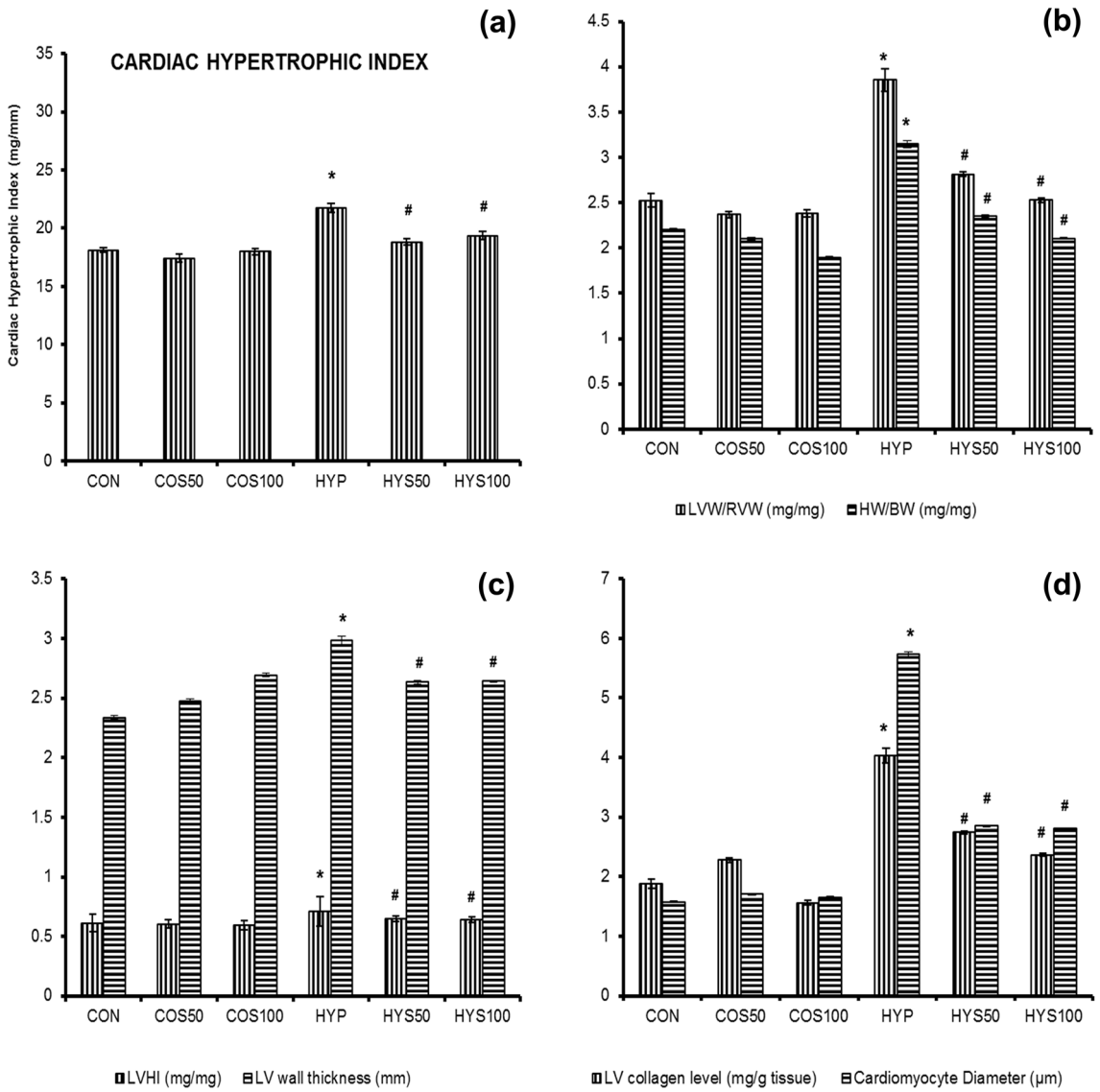

Fig. 1 Effect of silymarin on **a** cardiac hypertrophic index **b** LV weight-to-RV weight ratio and heart weight-to-body weight ratio **c** LV hypertrophic index and LV wall thickness **d** LV collagen levels and cardiomyocyte diameter. *Significantly different from normal control ($p < 0.05$). #Significantly different from hypertrophic control ($p < 0.05$). Each bar represents Mean ± S.E.M of six rats. CON—Sham control, COS50—Sham control animals treated with silymarin (50 mg/kg/day, p.o), COS100—Sham control animals treated with silymarin (100 mg/kg/day, p.o), HYP—hypertrophic control, HYS50—hypertrophic animals treated with silymarin (50 mg/kg/day, p.o), HYS100—hypertrophic animals treated with silymarin (100 mg/kg/day, p.o)

Table 1 Effect of silymarin on hemodynamic parameters

Parameters	CON	COS50	COS100	HYP	HYS50	HYS100
Blood pressure (mmHg)	117.64 ± 1.17	111.96 ± 3.65	113.65 ± 1.11	153.67 ± 0.69*	143.26 ± 2.71#	140.7 ± 1.94#
Heart rate (Beats/min)	241 ± 1.16	279 ± 1.12	260 ± 1.65	370 ± 1.37*	209 ± 1.46#	227 ± 1.02#
Rate of pressure development (%mmHg/sec)	96.63 ± 0.66	100.62 ± 1.06	100.44 ± 0.96	50.34 ± 2.31*	60.33 ± 1.50#	65.50 ± 1.49#
Rate of pressure decay (%mmHg/sec)	100.97 ± 2.90	101.15 ± 1.05	100.62 ± 1.25	49.25 ± 1.29*	75.60 ± 1.64#	62.36 ± 1.73#

Values represent Mean ± SEM of six experiments

*Significantly different from sham control ($p < 0.05$)

#Significantly different from hypertrophic control ($p < 0.05$)

COS 50—Sham treated with silymarin (50 mg/kg/day, p.o)

COS100—Sham treated with silymarin (100 mg/kg/day, p.o)

HYS50—Hypertrophic control treated with silymarin (50 mg/kg/day, p.o)

HYS100—Hypertrophic control treated with silymarin (100 mg/kg/day, p.o)

CON—Sham control

HYP—Hypertrophic Control

Serum Lipid Profile

PAAC control rats exhibited significantly increased serum total cholesterol, LDL, VLDL, triglyceride levels and log triglyceride/HDL ratio as compared to normal control rats. Administration of silymarin significantly decreased serum total cholesterol, LDL, VLDL and triglyceride levels and log triglyceride/HDL ratio in PAAC treated rats as compared to PAAC control rats (Table 2). There was a significant decrease in serum HDL levels in PAAC control rats which was significantly increased by silymarin treatment (Table 2).

Non-Specific Cardiac Markers

There was a significant increase in serum LDH, CK-MB and CRP levels and a significant decrease in LV Na^+K^+ATPase activity in PAAC control rats. Treatment with silymarin significantly reduced serum LDH, CK-MB and CRP levels and significantly increased LV Na^+K^+ATPase activity in PAAC treated rats as compared to PAAC control rats (Table 3).

Oxidative Stress Parameters

PAAC control rats showed a significantly increased LV MDA levels and significantly decreased LV SOD and LV GSH levels as compared to normal control rats.

Table 2 Effect of silymarin on lipid profile

Parameters	CON	COS50	COS100	HYP	HYS50	HYS100
Serum lipid profile						
Serum cholesterol (mg/dl)	111.12 ± 1.52	101.36 ± 2.11	95.57 ± 1.56	157.24 ± 1.62*	127.04 ± 2.20#	115.66 ± 2.30#
Serum LDL (mg/dl)	67.56 ± 1.96	57.63 ± 1.77	52.65 ± 1.26	114.51 ± 1.74*	76.16 ± 3.31#	67.55 ± 3.76#
Serum VLDL (mg/dl)	14.06 ± 0.65	12.32 ± 0.43	12.15 ± 0.65	23.74 ± 0.62*	17.79 ± 0.55#	17.31 ± 0.56#
Serum triglyceride (mg/dl)	71.64 ± 1.23	61.7 ± 2.19	57.56 ± 1.46	116.72 ± 2.37*	66.9 ± 2.74#	64.56 ± 1.94#
Serum HDL (mg/dl)	33.1 ± 1.06	30.16 ± 1.26	32.62 ± 0.65	21.7 ± 0.90*	25.6 ± 1.46#	26.6 ± 1.39#
Log TG/HDL	0.021 ± 0.0005	0.020 ± 0.001	0.017 ± 0.0004	0.054 ± 0.002*	0.025 ± 0.002#	0.032 ± 0.001#

Values represent Mean ± SEM of six experiments

*Significantly different from sham control ($p < 0.05$)

#Significantly different from hypertrophic control ($p < 0.05$)

COS 50—Sham treated with silymarin (50 mg/kg/day, p.o)

COS100—Sham treated with silymarin (100 mg/kg/day, p.o)

HYS50—Hypertrophic control treated with silymarin (50 mg/kg/day, p.o)

HYS100—Hypertrophic control treated with silymarin (100 mg/kg/day, p.o)

CON—Sham control

HYP—Hypertrophic control

Table 3 Effect of silymarin on non-specific cardiac markers

Parameters	CON	COS50	COS100	HYP	HYS50	HYS100
LDH (U/I)	667.66 ± 1.96	677.54 ± 1.62	656.96 ± 2.54	1491.54 ± 2.20*	619.12 ± 1.43#	613.62 ± 1.47#
CK-MB (U/I)	491.96 ± 3.40	467.44 ± 3.10	491.64 ± 3.0	655.67 ± 3.64*	979.32 ± 3.09#	640.16 ± 1.76#
CRP (mg/dl)	7.3 ± 0.69	6.6 ± 0.46	6.9 ± 0.51	16.62 ± 1.15*	15.26 ± 0.90#	14.16 ± 0.63#
Na^+K^+ATPase activity (nmoles pi liberated/hr/mg protein)	20.33 ± 2.27	16.44 ± 0.56	16.59 ± 0.67	7.62 ± 0.64*	16.76 ± 0.67#	16.90 ± 0.61#

Values represent Mean ± SEM of six experiments

*Significantly different from sham control ($p < 0.05$)

#Significantly different from hypertrophic control ($p < 0.05$)

COS 50—sham treated with silymarin (50 mg/kg/day, p.o)

COS100—sham treated with silymarin (100 mg/kg/day, p.o)

HYS50—hypertrophic control treated with silymarin (50 mg/kg/day, p.o)

HYS100—hypertrophic control treated with silymarin (100 mg/kg/day, p.o)

CON—sham control

HYP—hypertrophic control

Administration of silymarin produced a significant decrease in LV MDA and a significant increase in LV SOD and LV GSH levels PAAC treated rats as compared to PAAC control rats (Table 4).

Histopathological Analysis

The histopathological analysis of LV microsections from control and control treated rats showed no signs of injury and normal histology. LV microsections from PAAC control rats depicted apoptotic cardiomyocyte, eosinophils and extravasated RBCs. LV microsections of silymarin treated rats exhibited increased interstitial space and decrease in eosinophilia and extravasated RBCs (Fig. 2).

mtDNA Concentration

There was a significant reduction in mtDNA concentration in PAAC control rats as compared to normal control rats. Treatment with silymarin significantly increased the mtDNA concentration in PAAC-treated rats (Fig. 3a).

Docking

Among all proteins docked, silymarin showed maximum binding score with MAPK p38 as compared to other targets (Table 5). The docking score of silymarin with MAPK p38 was 77.64 while that of the standards nilotinib and SB-203580 was 126.92 and 62.68 respectively (Table 5; Fig. 4a). Silymarin showed 3 hydrogen bonding while

Table 4 Effect of silymarin on oxidative stress markers

Parameters	CON	COS50	COS100	HYP	HYS50	HYS100
LV MDA levels (n mol/mg protein)	2.02 ± 0.19	3.44 ± 0.11	3.65 ± 0.16	5.71 ± 0.21*	4.96 ± 0.23#	4.92 ± 0.19#
LV SOD levels (unit/min/mg protein)	4.56 ± 0.09	4.41 ± 0.16	4.56 ± 0.06	2.06 ± 0.11*	3.29 ± 0.26#	3.59 ± 0.049#
LV GSH levels (µg/mg protein)	2.47 ± 0.11	2.47 ± 0.26	2.35 ± 0.15	1.27 ± 0.06*	2.11 ± 0.23#	2.16 ± 0.19#

Values represent Mean ± SEM of six experiments

*Significantly different from sham control ($p < 0.05$)

#Significantly different from hypertrophic control ($p < 0.05$)

COS 50—sham treated with silymarin (50 mg/kg/day, p.o)

COS100—sham treated with silymarin (100 mg/kg/day, p.o)

HYS50—hypertrophic control treated with silymarin (50 mg/kg/day, p.o)

HYS100—hypertrophic control treated with silymarin (100 mg/kg/day, p.o)

CON—sham control

HYP—hypertrophic control

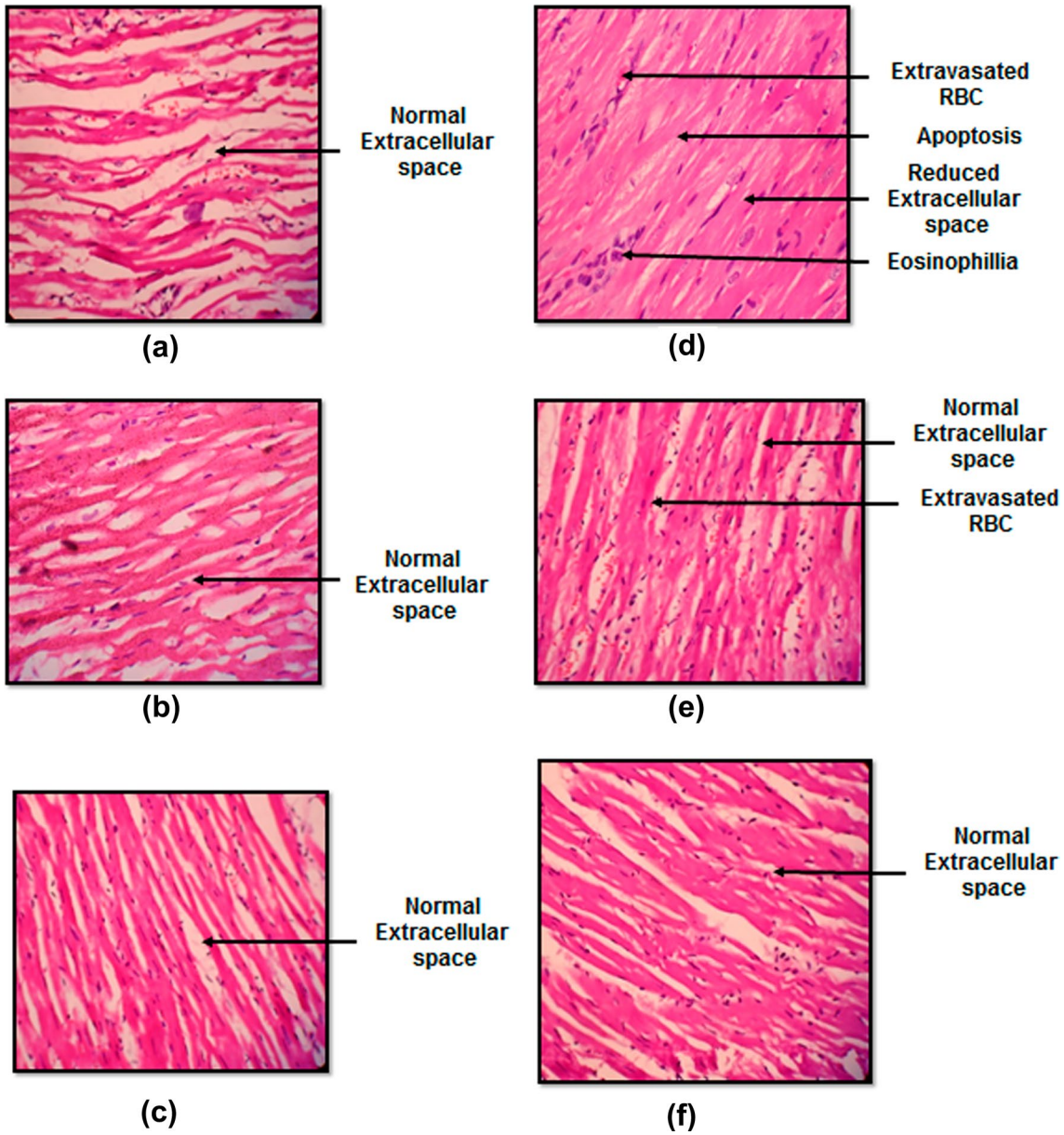

Fig. 2 Representative figure of left ventricular myocardial fibres (Magnification ×400) from **a** Sham control. **b** Sham control animals treated with silymarin (50 mg/kg/day, p.o), **c** sham control animals treated with silymarin (100 mg/kg/day, p.o). **d** Hypertrophic control. **e** Hypertrophic animals treated with silymarin (50 mg/kg/day, p.o). **f** Hypertrophic animals treated with silymarin (100 mg/kg/day, p.o)

nilotinib and SB-203580 showed 4 and 3 hydrogen bonding with the proteins (Fig. 4b–d). Silymarin exhibited strong hydrogen bonding with GLU/71 A and THR /106 A which is the same binding site for that of its standard nilotinib (Fig. 4c, d).

mRNA Expression of MAPK p38β

PAAC control rats exhibited a significant increase in MAPK p38β mRNA levels as compared to normal control rats. Treatment with silymarin significantly reduced MAPK p38β mRNA levels in PAAC treated rats as compared to PAAC control rats (Fig. 3b).

Kaplan–Meier Plot for Survival Probability

Kaplan–Meier analysis revealed that the survival rate reduced to 50% in PAAC control animals. However, administration of silymarin significantly improved the survival rate to 75% in hypertrophic rats (Fig. 3c).

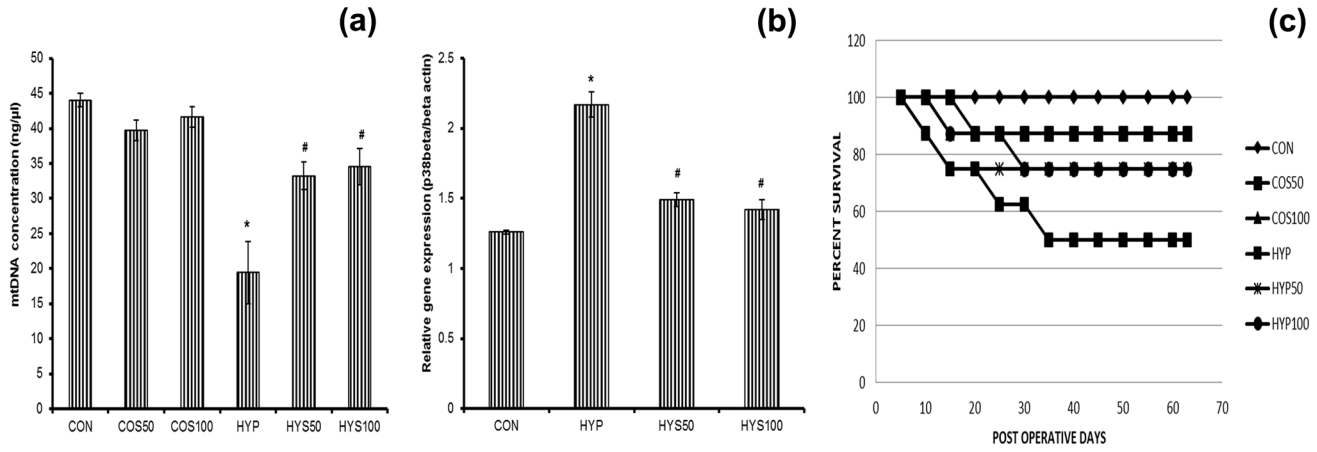

Fig. 3 Effect of silymarin on **a** mitochondrial DNA (mtDNA) concentration **b** MAPK p38β mRNA levels **c** percent survival. *Significantly different from normal control ($p<0.05$). #Significantly different from hypertrophic control ($p<0.05$). Each bar represents Mean ± S.E.M of six rats. CON—sham control, COS50—sham control animals treated with silymarin (50 mg/kg/day, p.o), COS100—Sham control animals treated with silymarin (100 mg/kg/day, p.o), HYP—hypertrophic control, HYS50—hypertrophic animals treated with silymarin (50 mg/kg/day, p.o), HYS100—hypertrophic animals treated with silymarin (100 mg/kg/day, p.o)

Table 5 Docking score and interactions of various targets with silymarin, SB-203580 and nilotinib

Target	DRUG	Goldfitness score	Docking interaction
Mitogen-activated protein kinase (MAPK) p38	SB-203580	62.68	GLU71, ASP168
Mitogen-activated protein kinase (MAPK) p38	Nilotinib	126.92	GLU71A, THR- 106A, MET-109A, ASP166-A
MAPK p38	Silymarin	77.64	ILE-141, THR-106A, GLU71A,c LEU-73
Calmodulin kinase II	Silymarin	54.28	VAL93, GLU97, ASP157
Mammalian target of rapamycin (mTOR)	Silymarin	72.30	ASP2195, ASP2357
Janus kinase (JNK)	Silymarin	62.32	MET149, ASP150
AkT	Silymarin	55.45	ASP292
B-Raf	Silymarin	65.11	LEU514
Extracellular signal-regulated kinases (ERK)	Silymarin	63.78	ARG65, GLU69, SER151
Histone deacetylase 2 (HDAC-2)	Silymarin	71.14	ARG39, GLY143, HIS145, PHE210, LEU276, TYR308
Histone deacetylase 8 (HDAC-8)	Silymarin	–	Silymarin did not fit in the binding pocket

Discussion

Cardiac hypertrophy, characterized by increase in cardiac myocytes volume, is one of the most important risk factors for chronic heart failure [12]. PAAC model is a pressure overload model which leads to activation of stretch sensitive ion channels, receptors and integrins which leads to activation of MAPK signalling pathway [5]. In the present investigation, PAAC-induced cardiac hypertrophy model exhibited significant increase in several hypertrophic indices and these hypertrophic indices were significantly decreased in the rats treated with silymarin. Activation of MAPK signalling pathway results in the activation of mitogen-activated protein kinase (MEKK) factors, which leads to activation of the three terminal MAPK effectors i.e. c-Jun NH2 terminal kinases (JNK1/2/3), extracellular signal-regulated kinases (ERK1/2), and MAPK p38 specially MAPK p38β [31]. Gharagozloo et al. [16] reported that the treatment with silymarin is able to inhibit ERK1/2 and MAPK p38 pathway in T cell proliferation in vitro study of CD4 + T cell. Thus, it is possible that silymarin through its inhibitory activity on MAPK p38 might have prevented hypertrophy in PAAC model.

Left ventricle has been associated with complications like atrial fibrillation, systolic and diastolic heart failure and left ventricular dysfunction [27, 39]. In the present study, PAAC control rats exhibited hypertension, bradycardia and decline in the rate of pressure development and decay, which are indicative of left ventricular dysfunction. Treatment with silymarin significantly improved the hemodynamics by controlling hypertension, bradycardia, and increasing the rate of pressure development and decay. It has been reported that activation of MAPK p38 leads to down-regulation of

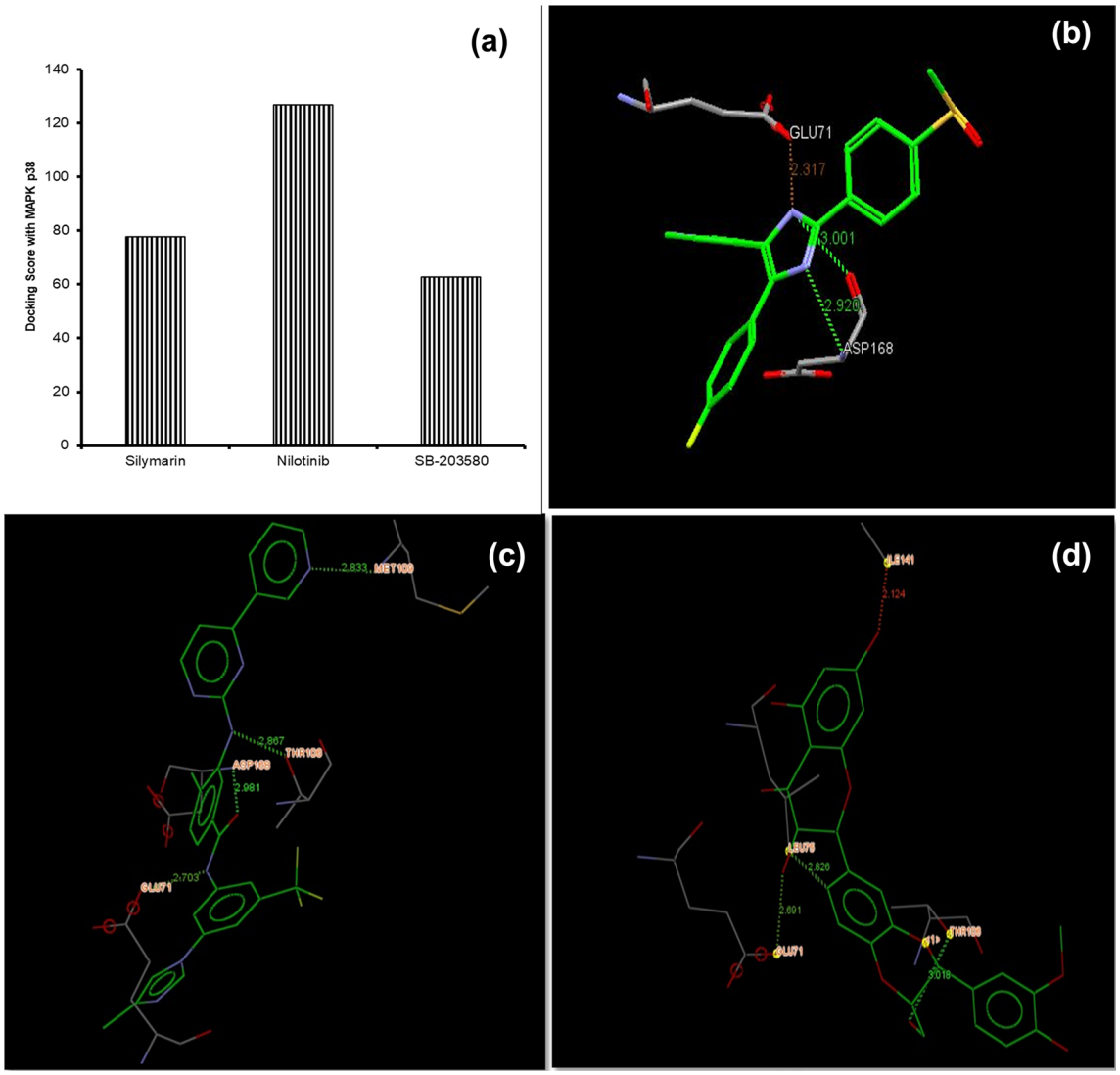

Fig. 4 **a** Docking score of silymarin and nilotinib with MAPK p38. **b** Number of hydrogen bonds. **c** Co-crystallized structure of Nilotinib with MAPK p38. **d** Docking of Silymarin on MAPK p38

SERCA2 which prolongs the decay of contractile response [2]. This supports the possibility that improved hemodynamics and contractility by silymarin in current study may be due to the modulation of SERCA2 by MAPK p38β inhibition.

Dyslipidemia is one of the most modifiable risk factors for CVDs. It has been reported that abdominal aortic banding worsens atherosclerosis in mice lacking apolipoprotein E possible because banding produces hypertension leading to increased intimal permeability [56]. In current study, PAAC control rats exhibited dyslipidemia and treatment with silymarin prevented dyslipidemia and improved the lipid profile. It is reported that silymarin can reduce the liver cholesterol synthesis by suppressing 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity [48]. This could be the possible mechanism behind prevention of dyslipidaemia by silymarin.

A significant increase level of LDH is found in case of myocardial damage due to hypertrophy [23]. Na^+K^+ATPase dysfunctioning has been found to be associated with myocardial contractile dysfunction [24]. In present study, there was a significant increase in serum CK-MB and LDH levels and a significant reduction in Na^+K^+ATPase activity in PAAC control rats and these cardiac biomarkers levels were improved by silymarin treatment. The decrease in the cardiac biomarker levels by silymarin indicates that silymarin is beneficial in preventing cardiac hypertrophy and thereby prevents cardiac damage.

Elevated levels of CRP are associated with traditional cardiovascular risk factors [7]. In the present investigation, CRP levels were found to be significantly increased in PAAC control rats and treatment with silymarin significantly reduced the CRP levels. Evidence has been accumulating

that the inhibition of MAPK p38 during prolonged ischemia inhibits the production of inflammatory cytokines, such as IL-6, which is known to aggravate ischemic injury [28]. It has been reported that silymarin inhibits the expression of the IL-6 signalling pathway-related genes in animal model of hepatotoxicity induced by CCl_4 [30]. Thus, it is possible that silymarin inhibits MAPK p38 which in turn inhibits IL-6 formation leading to decrease in CRP levels, in present investigation, suggesting prevention of inflammation.

It has been reported that increased generation of reactive oxygen species in myocardium results into increased oxidative stress and is associated with progression of cardiac hypertrophy and heart failure [11]. In present study, there was significant increase in pro-oxidant MDA levels and decrease in antioxidants i.e. GSH and SOD levels in LV. Treatment with silymarin significantly prevented this oxidative stress.

Depletion of mtDNA and its reduced replication have been identified as markers of transition from compensated hypertrophy to right ventricular failure [26]. In the present study, PAAC hypertrophic rats exhibited decreased LV mtDNA concentration which was increased with silymarin treatment. Reports suggest that oxidative stress leads to disruption of mitochondrial proteins in mtDNA mutation [8]. As mentioned above, in the present study, silymarin prevented oxidative stress, which might be the probable mechanism for increased mtDNA concentration in hypertrophic-treated animals. Additionally, it has been reported that depletion of mtDNA alone sufficient to produce cardiac hypertrophy [1, 10, 46] and hence increase in mtDNA by silymarin treatment suggest beneficial role in cardiac hypertrophy.

Improvement in cardiac hypertrophy was further evident by histopathological study of the transverse microsections of LV. Histopathological examination in PAAC control rats revealed, reduction of interstitial space, enlarged cardiomyocyte, eosinophilia, extravasated RBCs and apoptosis. Treatment with silymarin in hypertrophic-treated rats showed less reduction in interstitial space, less increase in cardiomyocyte diameter and reduction in eosinophilia, extravasation of RBCs and apoptosis as compared to hypertrophic control rats indicative of beneficial effect of silymarin.

From ongoing discussion, it is evident that silymarin produces beneficial effect on cardiac hypertrophy. In order to determine the mechanism behind cardioprotective effects; we carried out docking of silymarin on various targets of cardiac hypertrophy. We found that silymarin binding score with MAPK p38 was comparable and similar to that of its standard nilotinib. Silymarin and nilotinib exhibited strong hydrogen bonding with same amino acids i.e. GLU/71 A and THR /106 A. This similarity in the binding interaction is due to the presence of heteroatom like nitrogen and oxygen in nilotinib and silymarin, respectively. Moreover, both show same binding interaction due to the presence of loan pair of electron on nitrogen and oxygen. Further, it has been reported that ATP binding pocket in all type of kinases is considered to be the conserved region [3]. Docking results depict that silymarin shows interaction with Thr/106 A which is a part of ATP binding pocket. This suggests that silymarin interacts with the conserved region of the MAPK p38.

Considering the docking results which showed silymarin inhibit MAPK p38, we carried out mRNA expression studies of MAPK p38β from the rat heart in order to confirm the mechanism of action. We found that hypertrophic control rats depicted over expression of MAPK p38β mRNA levels. The MAPK p38 beta mRNA expression was reduced in the rat hearts treated with silymarin. Thus, although we could not measure the phosphorylation of p38β, we can still establish that silymarin inhibit MAPK p38β expression in the hypertrophic-treated animals.

It is important to evaluate the long-term efficacy and tolerability of Silymarin in order to translate the preclinical research into clinical practice. In lieu of this, we monitored the animals for 9 weeks in PAAC model, respectively, and plotted the Kaplan–Meier curve. Normal control rats showed 100% survival rate throughout the study but hypertrophic control rats showed up to 50% mortality rate. Treatment with the silymarin improves the survival and produced survival up to 75% suggesting that it is safe drug.

Many novel p38 MAPK inhibitors entered into clinical trials, but had been withdrawn due to their off target effects on liver and CNS [29]. In addition to hepatoprotective, silymarin is known produce beneficial effects in CNS disorders like depression [50–52] and is also neuroprotective in nature [6]. Hence, it can be considered for beneficial effect in cardiac hypertrophy. Currently, silymarin is clinically indicated for hepatic disorders like hepatitis, jaundice, liver cirrhosis, alcoholic liver disease etc. Results of the present investigation provide sufficient evidence that silymarin prevents cardiac hypertrophy and thus its transition to heart failure. Moreover, given the fact that silymarin is safe molecule since it is already used for hepatic disorders, it could be directly subjected to clinical trials. In future, long term clinical trials can be carried out in heart failure patients to determine effectiveness of silymarin as an adjuvant therapy to improve the morbidity and thereby increase the long term survival. Although we have carried out a detailed study, there are certain limitations of the present study. We could not perform echocardiography of the rats and, hence, were not able to determine certain cardiac functions like LVEDP, LVEDV etc. Moreover, we could not study the

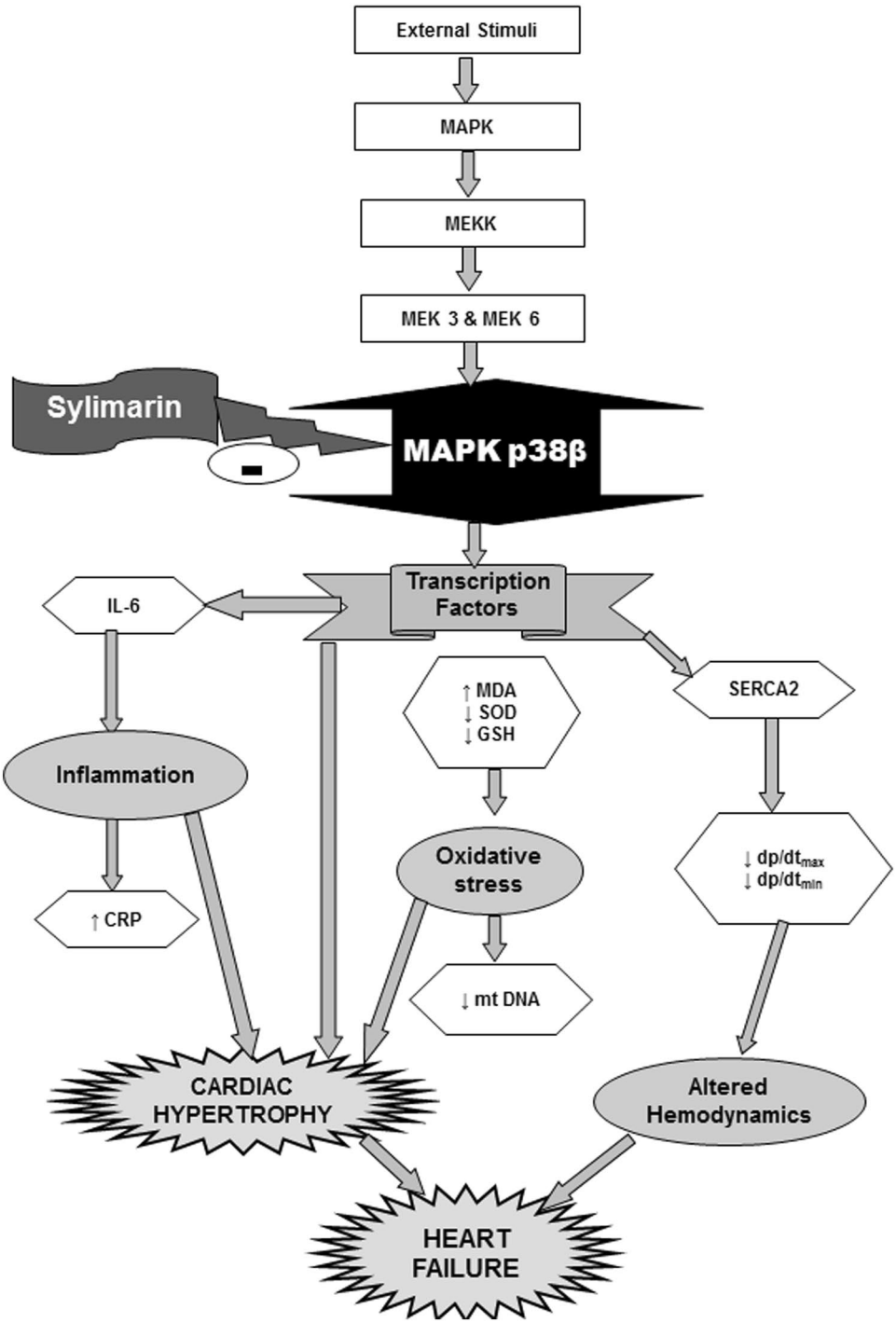

Fig. 5 Summarized effects of silymarin-mediated through MAPK p38

phosphorylated status of MAPK p38β and upstream targets of MAPK p38β to determine the exact pathway through which silymarin acts. In future, studies could be directed towards determination of phosphorylated MAPK p38β and MAPK p38β protein levels.

Conclusions

In conclusion (Fig. 5), our data suggest that silymarin produces beneficial effects on cardiac hypertrophy as evident from reduction in hypertrophic indices, preserving of hemodynamics and increase in mitochondrial DNA concentration. These effects of silymarin are possibly mediated through inhibition of MAPK p38β.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests.

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